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(1802 – 1880)

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Gerrit Jan Mulder [1] was born in Utrecht in 1802, the son of a surgeon, a modest profession in those days, not requiring academic training but mainly practical experience. His father was determined that his son follow the same profession and therefore directed his early education to this end. Anatomy was the principal theoretical basis of this education and osteology formed an integral part of it. Therefore human bones were among this boy's first playthings. At the age of 8 he performed his first bleeding. At the early age of 13 he was a "prosector" at a course for midwives in the *Theatrum anatomicum*, where he had to make the anatomical preparations.

Later on the professor of anatomy, B. F. Suerman, persuaded the parents to allow the young Mulder to study medicine at the University of Utrecht. They did this reluctantly, because they had to overcome some moral scruples in thus giving much more financial support to Gerrit than they could the other 4 children. They yielded, however, because Gerrit Jan excelled in zeal and intelligence; moreover, he was persevering and gifted with a phenomenal memory. Gerrit Jan thus became a student and was considered by his family as standing far above them. This probably had an unfavorable influence on the development of his character and personality. Although he entered the university in 1819 with scanty knowledge of Latin, mathematics and other subjects, he finished his studies in 1825 with a doctor's degree in medicine and in pharmacy.

[1] He is always called by this name. The name as given in the register of birth is GERARDUS JOHANNIS MULDER.

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During the following years Mulder lived the life of a physician in Rotterdam; in addition he became a teacher at the medical school in that town. He had to give lectures in chemistry, pharmacy, pharmacology, botany, materia medica, zoology, geology, mineralogy and physics! To perform this enormous task he worked nearly 20 hours a day, allowing himself only 4 hours for sleeping. The result was a severe collapse in 1835, and he had to give up his work for a long period.

This collapse, however, had a decisive influence on his future career. As a student he had attended lectures in chemistry for 6 years but he had not taken more interest in this branch of science than in any of the other branches. Moreover, he had had no practical experience in chemistry, because the training in the university had been only a theoretical one. During his recovery he applied himself more to practical work. In the beginning he had to surmount extreme difficulties and had to train himself in even the simplest chemical manipulations. When in difficulty he wrote to the famous Berzelius in Sweden, who always answered immediately. Elementary analysis presented him with extreme difficulties. For exercise he analyzed cane sugar again and again, but he had to repeat this task 100 times before getting reliable results. This serves to emphasize his great perseverance, if we remember that every analysis took not less than 7 hours.

Having overcome these basic difficulties, Mulder developed his ideas about protein chemistry, which gave him world fame. The result was his appointment in 1840 (with the strong support of Berzelius, Faraday and Liebig) as professor of chemistry in the University of Utrecht. During his professorship (1840 to 1868) his activities covered not only chemistry and education but also social hygiene, politics and the social position of the medical profession. He was a royalist and a convinced anti-liberalist. Because of illness he had to retire in 1868.

Mulder is said to have been emotional, irritable, inflexible, persevering and restless. He had devoted friends who idolized him, and he also had implacable enemies. In his later years,

because of his inflexible nature, he repelled many a good friend. After his retirement he lived in loneliness, separated even from his wife and children. Stricken with blindness and heart disease, he lived out the remainder of his days in a small village some 10 miles from the town of Arnhem. He died in 1880.

In modern scientific literature Mulder is usually mentioned in relation to his protein theory *(Over proteine en hare verbindingen en ontledingsproducten, Natuur- en scheikundig archief 6: 87, 1838,* and following papers). After heating several albuminous substances with dilute caustic soda he obtained, by neutralization with acid, a greyish-white precipitate. On the basis of his elementary analysis of this he thought it was always the same substance. He concluded, therefore, that the albuminous substances consist mainly of the same nucleus or root-substance, for which he proposed the name *proteine,* derived from the Greek *πρωτεῖος (primarius).* This word was suggested to him by the Swedish chemist Berzelius, to whom he expressed in the above paper his thanks for many valuable comments.

Of course Mulder did not overlook the fact that the various albuminous substances differed in their chemical and physical properties. In his opinion this could be explained by the union of protein with differing quantities of S and P (and O). In this way he arrived at the following formulas:

cristalline	= 15 protein + S
casein	= 10 protein + S
plant gluten	= 10 protein + S_2
fibrin	= 10 protein + SPh
albumin (hen's egg)	= 10 protein + SPh
albumin (blood serum)	= 10 protein + S_2Ph

As for protein itself, he proposed the formula $C_{40}H_{62}N_{10}O_{12}$. In calculating the N content of this compound, one will find it to be 16%, a figure that corresponds with the value used throughout the world for calculating protein from the N content. The ratio N : C amounts to 1 : 3.4, which also does not differ greatly from the ratio 1 : 3.25 now in use.

The fact that vegetable and animal egg white seemed to be essentially the same made a deep impression on his mind. The apparent great difference between vegetable and animal food seemed to be largely abolished, as is evident from the following quotations: "The animals eating only vegetable products eat the albuminous substance from the plants and this substance of which the muscle masses consist is taken immediately as such from the plants." "The grass-eating cows use similar food as the carnivores: both making use of the same egg white, one from plants and the other from animals; both are the same egg white." "It is highly admirable that the principal substance of all animals is immediately drawn from the plants. It signifies an economy of nature in her means that is wonderful and sublime." Many years later he expressed it this way: "A grass-eating cow and a lioness have similar meat, the same blood fibrin and albumin; lactating they give the same casein to their young; an infusorium in an extract of hay finds in this extract immediately the material for building up its organism and the pile worm that eats the wood gets the protein for its organism from the wood egg white, the latter undergoing only slight modification. Look here, there is a simplicity in this matter that is sublime and that fills one with admiration when one notes the boundless variety brought about by this single organic compound."

Mulder did not completely exclude the possibility that animals are able to build up protein from other substances. Nevertheless, he considered protein to be the foster substance of the whole animal kingdom, this substance being probably built up only by plants.

After these publications some people believed that the fundamental problems of life could be explained by protein. Mulder, however, criticized this view and stressed the necessity of extending experimental work greatly before such conclusion could be drawn.

Already in 1938 Mulder expressed the opinion that protein itself is a complex substance consisting of some heterogeneous organic compounds, leucine being one of them.

As for gelatin, he combatted vigorously the opinion of those days that it would be completely worthless as a food. As no protein could be prepared from gelatin in the laboratory, he readily admitted that it could not be used for building up new tissues; nevertheless he held that it might be useful for *maintaining* the organs. Indeed, his protein theory had important features.

The protein theory aroused general interest and in the beginning was highly appreciated by such leading chemists as Berzelius and Liebig. In vain Dumas claimed priority in the discovery; he was 4 years too late. McCollum and associates ('39) mention that Mulder's writings led Boussingault, in 1844, to assess the values of rations for farm animals largely on the basis of their nitrogen content.

In 1846, however, the storm broke as Liebig, who had many controversies with his contemporaries, contested the exactness of Mulder's analyses. A violent dispute followed, ending in Mulder's defeat. As a result the protein theory was no longer accepted, and Mulder's authority suffered a serious decline.

Looking at these questions from the vista afforded by the passage of 100 years, the situation seems less serious. Mulder's more general concepts continue to live in the amino acid theory. The amino acids in the animal kingdom and those in the plant kingdom are the same. The essential amino acids are built up by the plants and are used without modification for building the animal organism. The amino acid theory is therefore an extension of the protein theory. Moreover, in *practical* human and animal nutrition the differences among the various albuminous substances have been neglected for many, many years, as if these substances consisted only of Mulder's protein.

The fact is that Liebig had far better chemical training than Mulder. The latter, however, had essentially a medical training. It was, therefore, not difficult for Mulder to combat and to ridicule many of Liebig's primitive ideas on physiology

and medicine; and so he did in all the later years of his life. But his fame never returned. Moleschott, who knew both men personally, held them in equally high esteem.

Mulder's protein studies led him to consider problems of human nutrition, and in this field he developed excellent ideas, some of which could be appreciated fully only after the passage of 100 years. "Is there," he wrote, "a more important question for discussion than the nutrition of the human race?" In clear words he emphasized the importance of nutrition for the nation's physical and intellectual strength, necessary for full development of its capacity and activity in the competition of peoples. "With a faulty nutrition a nation remains on a low degree of development and is defective physically and mentally."

He was also fully aware of the fact that it is of little practical use to establish the minimum quantities of nutriments with which life is compatible. Just as in modern times, he aimed at a kind of nutrition that in our days would be called *optimum* — neither too little nor too much. "Considering the history of the diet of older and younger civilized nations, it appears that mankind and the human organism can endure much, but rules concerning the *best* kind of food are not given. I do not speak of such food which can preserve life and health. I mean such foods and beverages with which life and health are preserved in the best way." "Not to die, this cannot be the goal. It is life that I wish to promote."

From rations in use in the Netherlands army he arrived at a provisional nutrition standard in 1847; one of the first, possibly even the very first, ever published. It called for 100 gm of protein per day for a laborer; much less sufficed if the man was doing little work, for instance 60 gm or less per day. Many years later, on the basis of statistical data, Voit proposed a protein standard that was in general use for many years. It called for 118 gm per day. It was only after the researches of Chittenden, Hindhede and others that the high Voit standard was reduced to 50 to 100 gm a day, which

corresponds rather well with the figures arrived at by Mulder for soldiers in active service and in peace time.

Mulder also proposed a standard figure for carbohydrate intake. Here he combatted Liebig's erroneous opinion that carbohydrates and fats are used essentially for protecting the organs against the deleterious influence of the oxygen penetrating into the body by way of the lungs and through the skin. By the oxidation of the N-free substances the oxygen was rendered harmless. The heat produced was serviceable for maintaining body temperature. According to Mulder's ideas, however, carbohydrates as well as fats were essential for the metabolic functions, so that mutual substitution of fat and carbohydrate was only partly possible. "From carbohydrate and fat many substances are produced and in this way the heat production is kept going after these substances have performed various services in the body." He emphasized that a laborer eating much fat is able to work better.

Though on a feeble basis, Mulder came to the conclusion that 500 gm of starch a day (with some fat and the necessary salts) could be considered an appropriate quantity. Also in this respect he was about right, for many years later Voit advocated the same amount in his famous standard (118 gm protein, 56 gm fat, 500 gm carbohydrate). The quantity of fat, 56 gm, was probably somewhat higher than Mulder meant when he wrote "some fat."

In such an article as this it is impossible to give a full account of the prevailing ideas of those days and of Mulder's position regarding them. Although he was right many times, it cannot be denied that in other cases he was wrong. Still, the following comments are worthy of note. Notwithstanding the fact that vegetable and animal egg white did contain the same protein nucleus, Mulder did not consider them as fully equivalent. "Meat furnishes a stimulus — to use a general expression — that cannot be substituted by any plant protein compound." The grounds on which Mulder came to this conclusion seem ridiculous today. It appears that the history of the specific properties of animal protein food is a long one.

Mulder's activities were not restricted to questions of human nutrition. With his "Proeve ener algemene physiologische scheikunde," (Assay of General Physiological Chemistry, 1843–1850; 1,352 pages), he was one of the principal founders of this branch of science. He made extensive investigations into the composition of bile; he established the identity of caffeine and theine and introduced soda lime into elementary analysis. He studied the equilibria in the titration of silver nitrate, the chemism of drying oils and a great variety of other theoretical and technical problems.

In his restless activity he made an extensive study in the field of agricultural chemistry, evidenced by "De scheikunde der bouwbare aarde," (The Chemistry of the Cultivable Soil, 1860, 4 volumes; translated into German). Mulder defended the old humus theory against the mineral theory of Liebig. According to Mulder, it is the soil that regulates the reaction of the plant to fertilizers. The well-known conception that not the plant should be fertilized but rather the soil, originated in his mind. In brief, the ideas in the above-mentioned book are of such stature that Van Bemmelen, the famous father of colloid chemistry, regretted in 1901 that this book did not meet with more success, because it would have given a much better basis for further research than the theory of Liebig.

Notwithstanding the peculiarities of his character, Mulder was an unrivalled teacher. Irresistible was the impression he made on his pupils and they adored him. Education had his deep interest all his life. Formerly in the universities, practical exercises had been almost completely neglected. Like Liebig, he emphasized such practical work for students in general and for all students in chemistry in particular. Only three years after the foundation of the first laboratory for such practical work by Liebig in Germany, Mulder succeeded in establishing such a laboratory for his pupils in the Medical School in Rotterdam, and later on a second one for his stu-

dents in Utrecht. For this reason he is honored as the founder of modern chemical education in the Netherlands.

His endeavors in the field of education extended also outside the university. He advocated for instance the teaching of the principles of nutrition in the ordinary schools. The time was not ripe, however, for such revolutionary projects.

Mulder had his periods of glory and recognition and his periods of eclipse. It is only in our own century that the value of his work and the brightness of his spirit can be fully recognized.

E. BROUWER
Laboratory of Animal Physiology
of the Agricultural University
Wageningen, the Netherlands

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AVAILABILITY OF THE ESSENTIAL AMINO ACIDS IN COTTONSEED MEAL[1]

K. A. KUIKEN[2]

WITH THE TECHNICAL ASSISTANCE OF MARY TRANT

Department of Biochemistry and Nutrition, Texas Agricultural Experiment Station, Texas Agricultural and Mechanical College System, College Station

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Cottonseed meal is one of the more important vegetable protein concentrates available for farm animal feeding. The protein is of good quality. Its essential amino acid content compares favorably with that of soybean meal except in the case of lysine, where the values are approximately 4.3 and 6.2% of the protein, respectively (Lyman et al., '47). However, cottonseed meal contains more methionine than soybean meal and therefore it is desirable to use combinations of these products in mixed feeds.

The use of cottonseed products in farm animal feeds is complicated by the presence of a substance which is toxic to nonruminant animals. The toxic component has been identified and has been named "gossypol." It is a complex polyphenolic, dicarbonyl compound which is soluble in the common fat solvents (Bailey, '48). All of the gossypol of the seed is contained in a gelatinous capsule called a pigment gland.[3] The gland wall is resistant to mechanical rupture. Water, alcohols and ketones cause swelling and rupture of the gland. Solvents

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[2] Present address: The Buckeye Cotton Oil Company, Cincinnati, Ohio.

[3] The gossypol and pigment glands used in this study were kindly supplied by the Southern Regional Research Laboratory.

such as hydrocarbons, in which gossypol is soluble, may fail to remove the pigment from the seed because these solvents do not break the gland wall (Boatner et al., '46, '47a, b). As much as 1% of this pigment may be present in cottonseed meals (Bailey, '48).

When cottonseeds are heated in a manner which ruptures the pigment glands, the gossypol is discharged from the gland and variable amounts of the pigment are changed to an insoluble form referred to as bound gossypol (Carruth, '18, '47; Boatner et al., '47b). This binding of the gossypol renders it non-toxic (Withers and Carruth, '18; Lyman et al., '44). The uncombined form, referred to as free gossypol, is readily extracted with ethyl ether.

The acute toxicity of gossypol has been studied recently by Boatner et al. ('48), Castillon and Altschul ('50), Eagle et al. ('48, '50) and Lillie and Bird ('50). Chronic toxicity studies have been reported by Ambrose and Robbins ('51).

The technology of cottonseed processing has advanced to the point where non-toxic meals are available in limited quantity. However, by the expedient of controlling the quantities fed, extensive use of cottonseed meal is now being made in rations for swine. The problem of the industry is to detoxify the meal in an economical process which will not adversely affect the feeding quality of the protein. The present study was undertaken in order to provide more information on the relationship between processing variables and the feeding quality of the protein as judged by laboratory determination of essential amino acid availability by a rat feeding method. Swine feeding tests which have been conducted parallel with phases of the laboratory study will be reported by other workers.

EXPERIMENTAL

The techniques employed in selecting and feeding the animals and in collecting and analyzing feces have been described previously in detail (Kuiken and Lyman, '48). The techniques are adaptations of those developed by Mitchell and co-work-

ers ('24, '26) in their studies of protein digestion and metabolism. The essential change is that microbiological methods of amino acid analyses were employed in the present study to evaluate individual amino acid availability.

Groups of 5 rats, weighing initially from 55 to 60 gm, were fed a stock ration containing 2% Fe_2O_3 as a feces marker for a 24-hour period. They were then fed ad libitum for 7 days a ration which contained 10% protein supplied by the experimental cottonseed meal. Following this period they were again fed the marked stock ration for 24 hours. They were then fed a ration containing 4% whole egg protein for a period of 7 days to permit estimation of the metabolic fecal excretion of amino acids. All rations were adjusted to a crude fiber content of 2.5% by the addition of wood pulp cellulose.

Feces were collected from the individual animal and stored under ether until the feeding period was terminated. The samples for each group were then pooled and prepared for analyses. It was necessary to pool samples for each group in order to complete the large number of assays required in this study. This procedure was justified by the fact that individual animal data obtained for 6 different test samples (partially reported, Kuiken and Lyman, '48) rarely differed by more than 5%.

RESULTS AND DISCUSSION

The data are not subject to statistical analysis. The author attributes significance to availability differences greater than 5% on the basis of the following considerations: (1) duplicate determinations previously reported (Kuiken and Lyman, '48) for identical samples with different groups of animals differed by less than 5%; (2) data for a sample of hydraulic cottonseed meal fed with and without sulfasuxidine in the ration to different groups of animals (table 1) agree to better than 5%; (3) availability values have been obtained for each of the essential amino acids in highly digestible proteins such as roast beef (Kuiken and Lyman, '48), purified soybean and

cottonseed globulin (unpublished data) of $100 \pm 2\%$; (4) additional unpublished data indicate duplicate results which differ by less than 5% for a sample of zein which was tested with and without lysine supplementation.

The availability of the essential amino acids in samples of cottonseed meal of commercial or pilot plant origin is given in table 1. In the case of commerical cottonseed flour [4] and also the hydraulic meal, a marked variation in availability of the individual amino acids was noted. Lysine was only 64% available for the cottonseed flour sample, while arginine was 93% available. Other values ranged between these extremes, with valine, leucine and isoleucine among the lower values. Methionine availability was as low as 67% in the hydraulic product, but a value of 82% was obtained for methionine in the commercial cottonseed flour. A much more uniform amino acid availability is characteristic of the two types of hexane-processed meals and the gland-free preparation. The availability of lysine, which may be very critically related to animal growth when these products are fed, ranged from 84% to 89% in the latter cases. It is, therefore, evident that processing variables can appreciably alter the availability of the essential amino acids in cottonseed meals.

Availability experiments of this type are subject to the criticism that microorganisms in the intestine may alter the amino acid distribution of food residues as they pass through the intestinal tract. Such an effect might be of particular importance in cases where relatively large amounts of proteins are non-digestible. To evaluate this effect, hydraulic cottonseed meal was fed in a ration which contained 2% succinylsulfathiazole in lieu of an equal amount of starch. The drug was included in the stock ration of the animal for a period of 10 days prior to the experiment and was removed from the feces by acetone extraction prior to microbiological analysis. These data are also included in table 1, and indi-

[4] Proflo.

TABLE 1

Availability of the essential amino acids in cottonseed products of commercial or pilot plant origin

AMINO ACID	PRODUCT AND AMINO ACID AVAILABILITY							
	Proflo [1]	Hydraulic meal	Hydraulic meal with 2% sulfasuxidine in ration	Screw press meal [2]	Isopropanol-extracted meal [3]	Meal extracted with hexane, then methyl ethyl ketone [4]	Hexane-extracted "degossypolized" meal [5]	Gland-free meal [4]
	%	%	%	%	%	%	%	%
Arginine	93	95	93	95	95	98	97	97
Histidine	90	85	85	91	93	96	95	96
Isoleucine	78	72	71	80	80	84	83	86
Leucine	75	72	69	86	84	90	90	89
Lysine	64	71	71	79	79	88	84	89
Methionine	82	67	68	82	78	88	89	88
Phenylalanine	89	81	79	90	88	94	93	94
Threonine	77	72	72	84	81	86	91	85
Tryptophan	91	83	81	89	88	93	94	93
Valine	79	73	70	86	83	90	89	90
Total nitrogen	88	77	76	87	86	92	91	90

[1] Cottonseed flour, Traders Oil Mill Company.

[2] A special product prepared under direction of investigators at the Southern Regional Research Laboratory by the South Texas Cotton Oil Company. Not heated over 200°F. during the processing operation.

[3] Harris et al., '47.

[4] Prepared at the Southern Regional Research Laboratory.

[5] Product of the Buckeye Cotton Oil Company.

cate that the suggested effect of intestinal bacteria was negligible.

Two important processing variables have been considered as possible factors affecting amino acid availability in cottonseed products. The first is degree of heating, and the second is the relative extent of removal and binding of gossypol in the product. Table 2 presents data on the effect of heat treatment on the availability of the essential amino acids

TABLE 2

Effect of heat on the availability of essential amino acids in solvent-extracted cottonseed meal (Laboratory preparation)

AMINO ACID	HEAT TREATMENT AND AVAILABILITY				
	Raw [1]	Steamed 30 min.	Autoclaved 10 min.	Autoclaved 30 min.	Autoclaved 60 min.
	%	%	%	%	%
Arginine	99	99	98	98	98
Histidine	99	98	97	95	96
Isoleucine	91	91	89	87	88
Leucine	95	94	91	91	91
Lysine	92	92	89	88	90
Methionine	94	93	91	90	90
Phenylalanine	96	95	94	95	94
Threonine	92	93	90	91	91
Tryptophan	97	97	94	93	95
Valine	94	93	90	90	91
Total nitrogen	96	96	94	92	92

[1] Contained 0.006% free gossypol, 0.07% total gossypol.

in a sample of solvent-extracted cottonseed meal which was prepared in the laboratory with all processing conducted at room temperature. The product was prepared as follows: freshly rolled meats were extracted three times by stirring with hexane and filtering. This was followed by 5 batch extractions with ethyl ether, then three extractions with hexane containing 10% methanol, and a final washing with hexane. The air dried product contained 0.006% free gossypol and 0.07% total gossypol as determined by the methods of Pons

et al. ('49, '50). The crude protein content was 56.8% (N × 6.25). The data in table 2 indicate a high degree of availability for all of the essential amino acids in the product. Moist heating, as severe as autoclaving for 60 minutes at 15 lb. pressure, had only a minor effect on the availability of the amino acids studied.

The effect of heat on the nutritive value of protein-rich foods has recently been reviewed by Patton ('50) and Griswold ('51). The evidence indicates that one would expect

TABLE 3

Effect of heat on the essential amino acid content of cottonseed meal (All data calculated to 16% N)

AMINO ACID	HEAT TREATMENT AND AMINO ACID CONTENT					AVERAGE VALUES
	Raw	Steamed 30 min.	Autoclaved 10 min.	Autoclaved 30 min.	Autoclaved 60 min.	
	%	%	%	%	%	%
Arginine	12.19	12.08	12.26	10.71	10.65	11.58
Histidine	2.85	2.91	2.89	3.05	3.02	2.94
Isoleucine	4.11	4.04	3.92	3.98	3.98	4.01
Leucine	5.73	5.82	5.72	5.92	6.02	5.84
Lysine	4.35	4.47	4.45	3.99	3.93	4.24
Methionine	1.52	1.46	1.51	1.44	1.47	1.48
Phenylalanine	5.76	5.56	5.74	5.44	5.50	5.60
Threonine	3.56	3.52	3.63	3.25	3.25	3.44
Tryptophan	1.18	1.16	1.17	1.22	1.20	1.19
Valine	4.97	4.68	4.66	4.85	4.85	4.80

extensive destruction of lysine as a consequence of heating cottonseed meal in the autoclave. Should this occur, it is possible that availability determinations of the type conducted here would not reflect this destruction. However, the analytical data presented in table 3 indicate only a 10% reduction in lysine content as a result of the most severe heat treatment. Lysine in cottonseed meal is therefore not as sensitive to destruction by moist heat as is lysine in soybean meal (Reisen et al., '47). It is important to note that while the heat treatment applied in this experiment equalled in degree any treatment expected in commercial processing, the

chemical nature of the product at the time of heating may or may not have been comparable with that occurring in industrial practice.

Gossypol is a reactive alcohol which is known to combine with proteins and carbohydrates (Castillon and Altschul, '50). One would expect combination to occur between gossypol and the amino group of lysine, and the resulting effect

TABLE 4

Effect of heating in the presence of gossypol on the availability of the essential amino acids in solvent-extracted cottonseed meal (Laboratory preparation)

AMINO ACID	TREATMENT AND AVAILABILITY			
	Raw [1]	0.1% gossypol added		
		Raw [2]	Steamed 30 min.[3]	Autoclaved 10 min.[4]
	%	%	%	%
Arginine	99	99	98	98
Histidine	99	99	97	98
Isoleucine	91	92	89	91
Leucine	95	94	91	92
Lysine	92	93	90	89
Methionine	94	93	90	93
Phenylalanine	96	96	94	95
Threonine	92	95	90	92
Tryptophan	97	96	95	95
Valine	94	94	91	92
Total nitrogen	96	97	94	94

[1] Contained 0.006% free gossypol, 0.07% total gossypol.
[2] Contained 0.10% free gossypol, 0.18% total gossypol.
[3] Contained 0.01% free gossypol, 0.18% total gossypol.
[4] Contained 0.18% total gossypol.

might be to reduce lysine availability. In order to test this possibility an acetone solution of gossypol was added to the cottonseed meal used in the previous experiment, to supply a level of 0.1% gossypol. The acetone was evaporated at room temperature and the meal was then subjected to heat treatment. An identical experiment was conducted with cottonseed pigment glands as the source of gossypol. The data in tables 4 and 5 indicate that this treatment did not seri-

ously alter the availability of lysine or any other of the essential amino acids. However, the gossypol data in table 4 indicate that binding did occur.

Cottonseed meals frequently contain as much as 1% total gossypol. Since adequate quantities of gossypol were not available to permit its use at such high levels in these experiments, the effect of heat-binding of gossypol on amino acid availability in meals of high gossypol content was evalu-

TABLE 5

Effect of heating in the presence of pigment glands on the availability of the essential amino acids in solvent-extracted cottonseed meal (Laboratory preparation)

AMINO ACID	TREATMENT AND AVAILABILITY			
	Raw	Pigment glands equivalent to 0.1% gossypol added		
		Raw	Steamed 30 min.	Autoclaved 10 min.
	%	%	%	%
Arginine	99	99	98	98
Histidine	99	98	98	98
Isoleucine	91	91	91	90
Leucine	95	93	93	91
Lysine	92	91	90	92
Methionine	94	94	92	92
Phenylalanine	96	95	95	95
Threonine	92	93	92	92
Tryptophan	97	96	96	98
Valine	94	94	93	92
Total nitrogen	96	95	95	95

ated through use of a hexane-extracted meal. Hexane does not cause rupture of cottonseed pigment glands (Boatner and Hall, '46) and essentially all of the gossypol of the seed is retained in meals prepared by extraction with dry hexane at room temperature. The data presented in table 6 indicate a rather general but moderate reduction of amino acid availability in this case. Lysine availability was somewhat lower than is obtainable with low gossypol products, but the binding of large amounts of gossypol in this experiment did not

result in a very marked specific reduction of lysine availability.

Commerical hydraulic processing usually involves heating the meal in the presence of the seed oil. The oil is an excellent solvent for gossypol and might, therefore, alter its reactivity. The data reported in table 7 were obtained in an experiment designed to evaluate the effect of this processing

TABLE 6

Effect of high levels of free and bound gossypol on the availability of the essential amino acids in cottonseed meal

AMINO ACID	TREATMENT AND AVAILABILITY HEXANE-EXTRACTED PRODUCT		
	Raw [1]	Steamed 30 min.[2]	Autoclaved 10 min.[3]
	%	%	%
Arginine	96.2	94.3	94.8
Histidine	92.1	91.8	90.9
Isoleucine	84.1	83.6	78.9
Leucine	83.5	88.1	82.2
Lysine	84.4	80.3	79.8
Methionine	85.5	85.0	82.2
Phenylalanine	90.9	91.5	89.0
Threonine	84.3	84.7	83.1
Tryptophan	94.4	93.0	93.6
Valine	86.0	80.0	81.4
Total nitrogen	88.8	89.1	87.0

[1] Contained 1.16% free gossypol, 1.4% total gossypol.
[2] Contained 0.69% free gossypol, 1.4% total gossypol.
[3] Contained 0.60% free gossypol, 1.5% total gossypol.

factor. Two portions of gland-free meal were mixed with cottonseed oil [5] in the ratio of 2 gm of meal to 1 gm of oil. The equivalent of 1% gossypol was added to the oil in one case. The preparations were then autoclaved for 10 minutes, cooled, and extracted with hexane to remove the oil. The data indicate that lysine availability was greately reduced

[5] Wesson.

by treating cottonseed meal with gossypol under these conditions. Leucine, isoleucine, valine, threonine and methionine availabilities were also definitely reduced by this treatment. The general pattern was similar to that obtained with hydraulic cottonseed meals; however, the methionine value was much higher than that observed in hydraulic products. The heat treatment applied in this experiment was relatively

TABLE 7

Effect of heating gland-free cottonseed meal in the presence of Wesson oil and Wesson oil containing gossypol on the availability of the essential amino acids

AMINO ACID	TREATMENT AND AVAILABILITY GLAND FREE MEAL AUTOCLAVED 10 MINUTES		
	Without Wesson oil [1]	With Wesson oil [2]	With Wesson oil + gossypol [3]
	%	%	%
Arginine	99	98	96
Histidine	99	98	95
Isoleucine	92	89	79
Leucine	94	92	85
Lysine	95	93	77
Methionine	95	90	85
Phenylalanine	97	95	92
Threonine	94	91	84
Tryptophan	98	96	94
Valine	94	92	86
Total nitrogen	97	94	91

[1] Contained 0.02% free gossypol, 0.13% total gossypol.
[2] Contained 0.01% free gossypol, 0.13% total gossypol.
[3] Contained 0.02% free gossypol, 0.80% total gossypol.

mild as compared with commercial processing, and this fact probably accounts for the failure to obtain even lower values in all cases. Comparison with data in table 1 indicates some general improvement in amino acid availability as a result of heating the original gland-free meal. No destruction of amino acids or reduction of availability was apparent as a result of heating in the presence of gossypol-free cottonseed oil.

SUMMARY

1. The availability of the essential amino acids in cottonseed meal samples which were processed in various ways was determined by a rat feeding method.

2. Marked variation in individual amino acid availability was characteristic of commercial hydraulic cottonseed meal. Lysine and methionine were particularly low, with values of 64 and 67%, respectively. Lysine availability values as high as 85% were obtained with special solvent and gland-free meals.

3. The binding of gossypol as a result of heating cottonseed meal in the presence of either gossypol or pigment glands did not reduce amino acid availabilities appreciably unless cottonseed oil was also present.

4. Heat treatment as severe as autoclaving for one hour at 15 lb. pressure did not reduce the availability of the essential amino acids in cottonseed meal of low oil content.

5. Lysine was more sensitive to heat destruction than the other essential amino acids. About a 10% reduction in lysine content was observed when solvent-processed cottonseed meal was autoclaved at 15 lb. pressure for 60 minutes.

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DEPOSITION OF B VITAMINS IN NORMALLY DEVELOPING FETUSES AS EVIDENCE FOR INCREASED VITAMIN NEEDS OF THE RAT FOR REPRODUCTION

II. PANTOTHENIC ACID AND BIOTIN [1]

HARVYE LEWIS [2] AND GLADYS EVERSON

Nutrition Laboratory, Home Economics Research Department, Iowa Agricultural Experiment Station, Iowa State College, Ames

TWO FIGURES

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The rate of transfer of thiamine and riboflavin from the mother to the developing young during the course of the gestation period in the normal rat has been reported by Barrett and Everson ('51). This study revealed a rapidly changing need for both B vitamins as pregnancy progresses in the rat, and suggested that more attention needs to be given to the vitamin intake of this species during the final days of pregnancy, when the synthesis of fetal tissues is proceeding at maximum speed. Data of a similar nature have now been completed for pantothenic acid and biotin, two vitamins needed in entirely different amounts for successful reproduction in animals.

There are many articles in the literature which indicate an unusually high need for pantothenic acid by young animals

[1] Journal paper J-1986 of the Iowa Agricultural Experiment Station, Ames, project 1061. A preliminary report, including the data on pantothenic acid, appears in the Proceedings of the Federation of American Societies for Experimental Biology, vol. 9, no. 1, 1950.

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[2] Present address: Louisiana Experiment Station, Baton Rouge.

and which report this vitamin to be an essential factor for satisfactory reproduction in the rat (Unna, '40; Henderson, McIntire, Waisman and Elvehjem, '42; Nelson and Evans, '46; Boisselot, '48). The present investigation, describing the day-by-day transfer of pantothenic acid to developing rat fetuses, reveals an unusual demand for this vitamin during the reproductive cycle of the rat; in fact, the quantity of pantothenic acid deposited in the young at term exceeds the amount present in the entire body of the adult female if the litter contains 8 to 10 young of normal birth weights.

Considerably less information is available regarding the importance of biotin during the intra-uterine life of the rat. Everson and associates ('48) observed that litters of stock animals composed of 9 or more young of normal size contained between 4 and 5 μg of biotin at birth, an amount equal to approximately one-fourth that present in the adult female. Possibly biotin synthesis is sufficiently great under certain conditions, however, to reduce the dietary needs for this factor considerably.

EXPERIMENTAL

Details of the experimental procedure used in the present investigation, the description of the animals, and the rate of growth of placental and fetal tissues were similar to those reported in full in paper I of this series (Barrett and Everson, '51). Briefly, the study involved measuring the quantities of pantothenic acid and biotin present in the fetal, placental, and maternal tissues of the rat at daily intervals from the 13th day of pregnancy to term. Three females and their litters, which in all cases included 9 or more young, were assayed at each interval of the reproductive cycle. The animals represented healthy stock females fed a modified Steenbock stock ration. The animals were sacrificed during their second pregnancies.

The pantothenic acid and biotin concentrations in the tissues were determined by the microbiological method, using *Lactobacillus arabinosus* as the test organism. All tissues for

pantothenic acid assay were treated with chicken liver enzyme and intestinal phosphatase. Samples for biotin assay were hydrolyzed with 4N H_2SO_4 for two hours at 15 pounds' pressure.

RESULTS

Good reproductive performance was observed for the experimental animals included in the study. An average of 8.5 young, with birth weights averaging 4.9 gm, were born to females in first pregnancies. With very few exceptions these young were active and normal in appearance.

Growth of placental tissue during second pregnancies was continuous throughout the entire reproductive cycle. A spurt in weight was observed between the 14th and 16th days of pregnancy, after which the increase in weight appeared to be more gradual. A maximum average weight of 7.4 gm was noted for the placentae per litter of young immediately before parturition.

Fetal tissues comprised a very small mass prior to the 16th day of pregnancy. Subsequently there was a very rapid growth of the fetuses; in fact, synthesis of these tissues was sufficiently great during the final 4 days of pregnancy to increase the weight of the litters from 13 to 55 gm.

Deposition of pantothenic acid in the various tissues

The concentrations of pantothenic acid in the maternal carcass and liver appeared to be unchanged throughout the reproductive cycle when the females had access to the customary stock diet, which at that time provided between 500 and 600 μg of pantothenic acid per day (table 1). Approximately 10 times as much pantothenic acid was present in hepatic tissue as was found in carcass tissues. The quantity of pantothenic acid in placental tissue was small; the placentae contained the lowest concentration of this vitamin among the tissues analyzed. Synthesis of placental tissues, therefore, created only a negligible need for pantothenic acid, since less than 40 μg of the vitamin could be accounted for in these tissues at term.

TABLE 1

Concentration of pantothenic acid in fetal, placental, and maternal tissues

PERIOD OF PREGNANCY	CARCASS		LIVER		PLACENTAE		FETUSES		
	Per gm	Total	Per gm	Total	Per gm	Total	Number studied	Per gm	Total
days	*μg*	*μg*	*μg*	*μg*	*μg*	*μg*		*μg*	*μg*
Full term	7.0	1,003	64	613	..	..	11	36	1,947
21	6.7	960	66	590	4.0	29	10	34	1,647
20	6.2	957	73	750	4.8	32	11	29	995
19	7.1	1,059	69	659	4.4	29	10	32	574
18	6.9	999	68	616	4.9	26	10	25	336
17	6.9	1,006	74	708	6.9	33	10	27	197
16	7.6	1,177	69	736	5.8	30	11	14	79
15	7.6	1,057	64	624	4.7	17	11	8	22
14	7.4	1,070	66	615	3.6	7	10	11	22
13	7.2	1,104	71	633	2.9	6	12	7	8
12	7.0	1,038	66	651	..	..	13	..	.
11	6.8	995	64	625	..	..	13	..	..
10	7.5	1,039	69	627	..	..	12	..	..
6	7.4	990	64	550	..	..	..	..	..

TABLE 2

Concentration of biotin in fetal, placental and maternal tissues

PERIOD OF PREGNANCY	CARCASS		LIVER		PLACENTAE		FETUSES		
	Per gm	Total	Per gm	Total	Per gm	Total	Number studied	Per gm	Total
days	*μg*	*μg*	*μg*	*μg*	*μg*	*μg*		*μg*	*μg*
Full term	0.076	10.9	0.98	9.3	...	...	11	0.106	5.80
21	0.073	10.4	1.04	9.2	0.05	0.36	10	0.113	5.54
20	0.079	11.8	1.01	10.3	0.06	0.39	11	0.071	2.46
19	0.061	9.0	0.99	9.6	0.06	0.42	10	0.053	0.97
18	0.079	11.4	1.07	9.7	0.04	0.20	10	0.053	0.72
17	0.065	9.5	1.07	10.1	0.04	0.23	10	0.056	0.40
16	0.065	10.1	1.01	10.6	0.04	0.21	11	0.054	0.31
15	0.065	9.0	0.95	9.4	0.04	0.15	11	0.026	0.07
14	0.069	9.9	0.98	9.2	0.05	0.11	10	0.025	0.04
13	0.065	9.9	1.08	9.6	0.03	0.05	11	0.020	0.02
12	0.079	11.8	0.96	9.4	...	...	13	....	...
11	0.073	10.9	0.96	9.4	...	...	13	....	...
10	0.065	9.0	1.08	9.9	...	...	12	....	..
6	0.069	9.3	1.02	8.6	...	...	..	....	...

The quantity of pantothenic acid present in the fetuses, on the other hand, was unusually high. In comparison to the amounts present in maternal tissues, the fetuses were very rich sources of the vitamin. As was mentioned earlier, at term the total pantothenic acid present per litter of young exceeded the amount found in the entire body of the adult female.

Deposition of biotin in the various tissues

Biotin assays of the maternal tissues revealed no tendency for depletion or accumulation of this factor throughout the second reproductive cycle of the stock rat. The females had access to nearly 2.5 μg of biotin per day from the stock ration. As will be noted in table 2, the livers of the mothers contained as much biotin as was present in the remainder of the animal body. The concentration of biotin in placental tissue did not change appreciably during the gestation period, and the day-by-day needs for this factor were not greatly influenced by development of these structures. The maximum amount of biotin present in the placentae was observed during the latter portion of pregnancy and at no time did these tissues account for more than 0.5 μg of the vitamin.

The concentration of biotin in the fetuses, which was 0.02 μg per gram of fresh tissue on the 13th day of pregnancy, increased 5-fold prior to parturition. Due to the unusually rapid synthesis of fetal tissues during the final 4 days of pregnancy, the total quantity of biotin present per litter increased from 0.72 μg on the 18th day of pregnancy to nearly 6.0 μg at term.

DISCUSSION

Figures 1 and 2 illustrate the extent to which pantothenic acid and biotin requirements were increased during reproduction when the need was judged by the deposition of these two factors in fetal tissues of different ages. Maximum need for both vitamins appeared to be present between the 20th and

21st days of the gestation period, when approximately 600 μg of pantothenic acid and 3.0 μg of biotin were transferred from the mother to the fetuses within 24 hours. Before this time the need for the two vitamins was considerably smaller, and prior to the 14th day of the gestation period the demands for

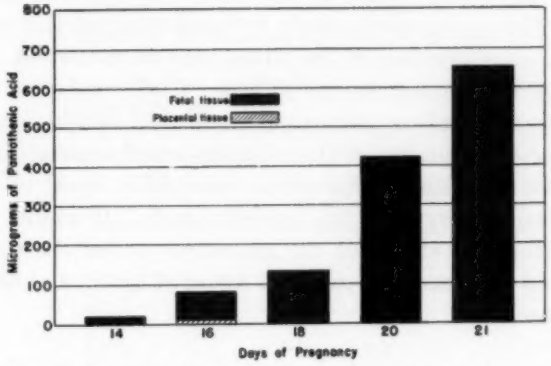

Fig. 1 Increased pantothenic acid needs of the rat during the second reproductive cycle, based on tissue analyses.

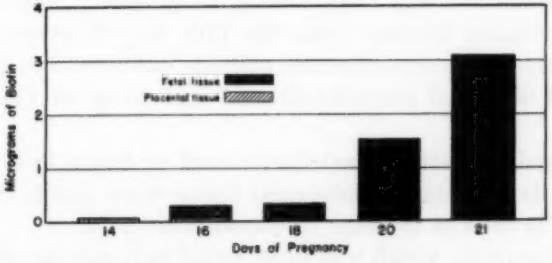

Fig. 2 Increased biotin needs of the rat during the second reproductive cycle, based on tissue analyses.

biotin and pantothenic acid above those of the non-pregnant animal were negligible.

These findings are of interest in view of the quantity of B vitamins frequently included in partially synthetic diets fed rats during reproduction. In many cases the quantities of

vitamins supplied daily by these diets have not been as great as the amounts transferred to the developing young in the present study. Just what influence these smaller intakes have on the reproductive performance is uncertain. Possibly maternal depletion occurs as the vitamin supply is limited, thereby altering the performance of the female during the lactation period. The young may be born with suboptimum stores of the vitamin, or a portion of the litter may be resorbed. These problems need study in the future. Until evidence is presented to the contrary, it would seem that synthetic rations should provide intakes of the vitamins ample enough to allow deposition of essential factors in quantities equal to the requirements observed in the present study. It would appear that during late stages of the gestation period the pregnant rat should have access to at least 3.0 μg of biotin and 600 μg of pantothenic acid daily.

SUMMARY

Information has been obtained on the daily transfer of pantothenic acid and biotin from the pregnant stock rat to the developing fetuses, from the 13th day of pregnancy to parturition. The experimental animals had access to between 500 and 600 μg of pantothenic acid and 2.5 μg of biotin per day.

Demands for either pantothenic acid or biotin to allow for normal development of placental tissue were small; this was especially true in the case of pantothenic acid.

Fetal growth, which was most rapid between the 20th and 21st days of the gestation period, accounted for an increased need for pantothenic acid of 600 μg per day. The highest requirement for biotin occurred during the same interval of the reproductive cycle and the rate of deposition of biotin at that time exceeded 3.0 μg per 24 hours.

No evidence of depletion of maternal tissues was encountered throughout the reproductive cycle.

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THE RIBOFLAVIN REQUIREMENT OF THE HOLSTEIN CALF

HAROLD H. DRAPER AND B. CONNOR JOHNSON
Division of Animal Nutrition, University of Illinois, Urbana

ONE FIGURE

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Since the development of a "synthetic milk" diet for calves by Wiese, Johnson, Mitchell and Nevens ('47a), several reports have been published from this laboratory in which studies of the vitamin nutrition of the young ruminant have been described. The same investigators ('47b) demonstrated a need for riboflavin by the dairy calf and reported the characteristics of the deficiency syndrome. In the present paper the results of a study designed to determine the quantitative requirement of the Holstein calf for this vitamin are described.

EXPERIMENTAL

The experimental animals were 12 purebred Holstein male calves which had previously received colostrum for one to three days following birth. Except for weekly 24-hour periods during which they were placed in metabolism cages to permit collection of urine samples, the calves were maintained throughout the experimental period of 10 weeks in metal cages 5×6 feet in size with raised floors constructed of heavy wire mesh.

Previous experience with calves maintained under these conditions had revealed the danger of pyloric obstruction as a consequence of swallowing loose hair licked from the haircoat. This hair accumulated in the rumen in the form of hairballs which eventually lodged in the pylorus and prevented

the passage of food into the intestine. This difficulty, and the possibility that the hairballs might serve as a site of bacterial synthesis of vitamins, was obviated by providing the animals with muzzles constructed of wire mesh trimmed with leather. The muzzles permitted the calves to drink from pails without interference but prevented them from licking their haircoats.

TABLE 1

Composition of synthetic diet

CONSTITUENTS	AMOUNT (dry basis)
Casein ("vitamin-free")	30 %
Lard	30 %
Mineral mixture [1]	6 %
Cerelose	34 %
Water-soluble vitamins	
Thiamine hydrochloride	0.65 mg per liter
Riboflavin	1.30 mg per liter
Calcium pantothenate	1.95 mg per liter
Nicotinic acid	2.60 mg per liter
Pyridoxine	0.65 mg per liter
Choline	260.0 mg per liter
Folic acid	0.26 mg per liter
Biotin	0.02 mg per liter
Fat-soluble vitamins	
Vitamin A	2000 I.U. per liter
Vitamin D	200 I.U. per liter
Alpha-tocopherol	1.0 mg per liter
2-Methyl-1,4-naphthoquinone	0.26 mg per liter

[1] For composition of mineral mixture see Johnson, James and Krider ('48).

The composition of the diet is shown in table 1. The casein, minerals and cerelose were dissolved in hot water to which sodium bicarbonate had previously been added (1.75 gm per liter). The lard and fat-soluble vitamins were homogenized into the solution so as to produce a "milk" containing 13% solids, and the complete diet was then pasteurized. Later in the experiment it was found convenient to increase the dry matter content to 19.5%, and a compensating reduction was made in the level of feeding. The water-soluble vitamins were

added in a 30% ethanol solution at the time of feeding, and a single dose of 100,000 I.U. of vitamin A plus 10,000 I.U. of vitamin D was administered to each calf at the beginning of the experimental period. Riboflavin was provided at levels ranging from 0.3 to 1.80 μg per gram of dry matter consumed. Sulfathalidine was supplied at the rate of 3 gm per liter of diet to inhibit the possible synthesis of riboflavin by microorganisms in the digestive tract. Previous experience with feeding calves "synthetic milk" has shown that the presence of this antibiotic in the fiber-free liquid diet has been associated with a marked underdevelopment of the rumen, greatly depressed bacterial counts in the feces, and early development of deficiency symptoms when one of several B vitamins was eliminated from the diet.

All animals were fed twice daily at a rate equivalent to 4.5 l of 13% "milk" per 100 lb. body weight per day, with the exception of one calf which, as is indicated later, was fed the 19.5% diet ad libitum. Free access to water was permitted, provided that the allotment of diet was consumed previously. The calves were weighed at weekly intervals and daily observations were made on their general condition.

RESULTS AND DISCUSSION

The growth data obtained from the 12 calves are presented graphically in figure 1, and a summary is shown in table 2.

Figure 1 illustrates the fact that the two lowest levels of riboflavin, 0.30 and 0.75 μg per gram of dry matter intake, were inadequate to support continued growth. Both calves on the latter level showed distinct growth responses when the riboflavin content of the diet was increased to 3.30 μg per gram. Despite progressive increases in riboflavin intake, no improvement in growth was obtained at levels beyond 1.05 μg per gram in any of the calves on a controlled food intake. However, growth in none of these animals was satisfactory when compared to tables of growth rates for the male Holstein calf as compiled by Morrison ('36), which indicate that a gain of 1.25 lb. per day may be regarded as average growth

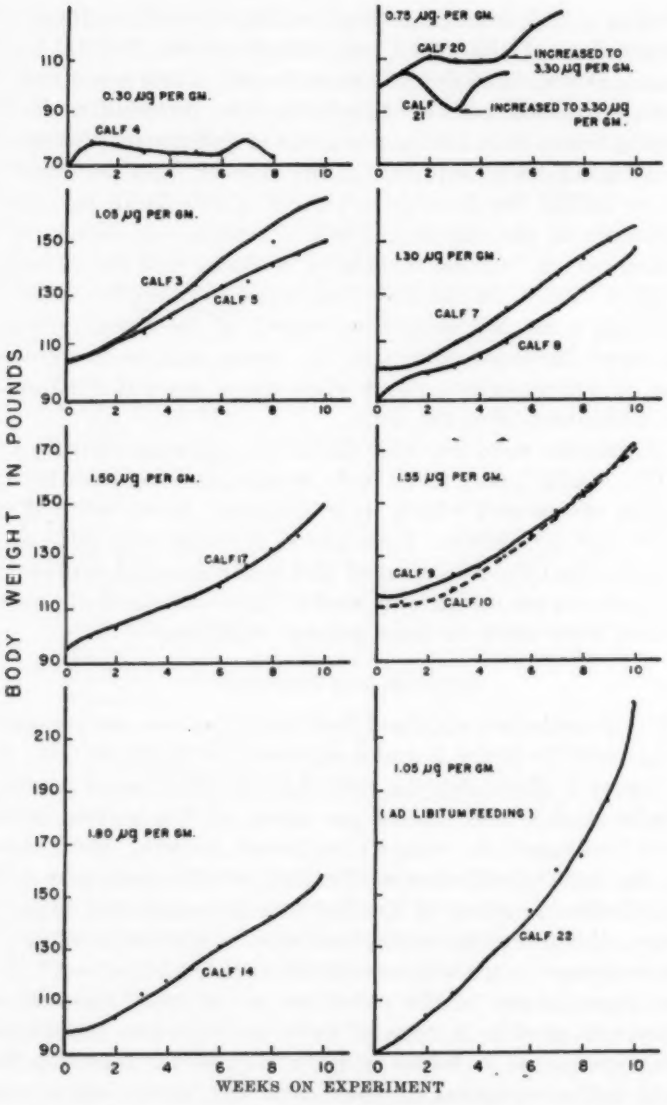

Fig. 1 Growth curves of calves maintained on different levels of riboflavin. Riboflavin content is expressed as micrograms per gram of dry matter in the diet.

during the first 10 weeks of life. Since the primary limitation on growth appeared to be the restricted level of food intake, calf 22 was maintained on ad libitum consumption at the critical riboflavin level of 1.05 μg per gram. It is apparent from figure 1 that this animal exhibited excellent growth (1.91 lb. per day), especially during the final two weeks of the experimental period. The voluntary dry matter intake averaged approximately 2.5 times that of the calves fed the

TABLE 2

Summary of growth rates and urinary riboflavin excretion of calves maintained on different levels of riboflavin in the diet

CALF NO.	RIBOFLAVIN LEVEL	AVERAGE DAILY GAIN	AVERAGE RIBOFLAVIN EXCRETED [2]
	μg/gm. dry matter	*lb./day*	*mg/24 hr.*
4	0.3	0	0.06
20	0.75	0	0.17
21	0.75	*weight loss*	—
3	1.05	0.89	0.26
5	1.05	0.66	0.25
7	1.30	0.76	0.24
8	1.30	0.83	0.24
17	1.50	0.79	0.15
9	1.55	0.81	0.46
10	1.55	0.87	0.21
14	1.80	0.86	0.25
22 [1]	1.05	1.91	—

[1] Fed ad libitum. All others fed at level of 4.5 l per 100 lb. body weight per day.
[2] Omitting excretion for first week.

restricted diet. The composite growth data indicate, therefore, that the minimum riboflavin requirement of the Holstein calf is approximately 1.0 μg per gram of dry matter in the diet.

Other criteria of adequacy were of little value in estimating the required riboflavin intake. Calf 4, maintained on 0.30 μg per gram, exhibited gross symptoms including a very rough haircoat, emaciation and listlessness, but did not show evidence of denudation, excess salivation or other symptoms

reported by Wiese et al. ('47b) in riboflavin-deficient Guernsey calves. The deficiency syndrome appears to differ significantly in this respect between the two breeds. Limited refusal to consume the allotted quantity of diet was encountered in calf 4 during the advanced stages of the experimental period. This was the only instance during the experiment in which the prescribed level of food intake could not be maintained. No further evidence of abnormality besides poor growth was observed in any of the other animals and histopathological examination of those animals on the lower levels of riboflavin failed to reveal any significant lesions.

The average riboflavin excretion of the calves showed little correlation with riboflavin intake. Riboflavin in the urine was estimated by the method of Najjar ('41). Calf 9 excreted a relatively uniform quantity of the vitamin, amounting to almost twice that excreted by any other animal. Moderate diarrhea was encountered in calf 22, fed ad libitum, and prohibited the collection of urine samples.

Recently Brisson and Sutton ('51) reported that dairy calves fed a basal diet of photolyzed milk required 35 to 45 μg of riboflavin per kilogram of body weight per day for the prevention of all deficiency symptoms. This finding is approximately three times the value arrived at in the present experiment. The effect of breed differences on the requirement may account, at least in part, for this discrepancy. Most of the animals employed by the aforementioned investigators belonged to the smaller breeds in which the deficiency syndrome, as noted earlier, is considerably more severe than in the Holstein.

SUMMARY

Twelve male Holstein calves were employed in a study of the riboflavin requirement during the first 10 weeks of life. Riboflavin levels were tested over a range of 0.30 to 1.80 μg per gram of dry matter in a "synthetic milk" diet containing sulfathalidine. When fed at the rate of 4.5 l of diet per 100 lb. body weight per day, no increase in growth rate was obtained

at any level beyond 1.05 μg per gram of dry matter. Excellent growth was observed in a calf fed the diet ad libitum at this level of riboflavin. The results indicate that the riboflavin requirement of the Holstein calf up to 10 weeks of age is approximately 1.0 μg per gram of dry matter consumed.

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BIOLOGICAL AVAILABILITY OF CERTAIN FOODS AS SOURCES OF RIBOFLAVIN [1]

GLADYS EVERSON, ELINOR PEARSON [2] AND ROBERTHA MATTESON [3]

The Nutrition Laboratory, Home Economics Research Department, Iowa Agricultural Experiment Station, Ames, and The Bureau of Human Nutrition and Home Economics, U.S. Department of Agriculture, Washington, D.C.

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Earlier studies relating to the availability of the riboflavin in foods have led us to believe that healthy young women absorb quite different proportions of the riboflavin present in various food sources (Everson, Wheeler, Walker and Caulfield, '48; Baker, '47). The bioassay used in the past to judge the availability of the riboflavin present consisted of comparing the urinary excretion of the vitamin when 1-mg quantities were supplied from either green peas, ice cream, soybeans, almonds, or a solution of pure riboflavin. To furnish this amount of the vitamin, it was necessary that the subjects consume large amounts of certain of the foods. We have therefore wondered whether incomplete digestion accounted for the differences observed in the quantity of riboflavin which was excreted in the urine when the young women consumed the test foods. These findings have aroused our interest in determining how efficiently such foods are digested when they are consumed in amounts in line with everyday eating practices. To date, relatively little is known as to how seriously

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[2] Present address: Georgia Experiment Station, Experiment, Georgia.

[3] Present address: Sacramento State College, Sacramento, California.

incomplete digestion reduces the value of certain foods as sources of vitamins.

EXPERIMENTAL

Four women subjects between the ages of 22 and 28 participated in a series of five 9-day metabolism studies planned to determine how thoroughly young women digest peas, ice cream, soybeans or almonds. One individual (GB) had also

TABLE 1

Basal diet

BREAKFAST		LUNCH		DINNER	
Food	Amount	Food	Amount	Food	Amount
	gm		*gm*		*gm*
Orange juice (canned)	135	Beef and ham patty	90	Beef and ham patty	90
Cornflakes	14	Lima beans (canned)	90	Tomatoes (canned)	135
Milk	180	Whole wheat bread	27	Rice (dry weight)	27
Whole wheat bread	27	Peaches (canned)	115	Whole wheat bread	27
Scrambled egg	45	Peaches (juice)	25	Milk	180
Coffee		Milk	180	Ice cream (vanilla)	90
		Cookies (macaroon)	27	Coffee	
				Sugar (per day)	18
				Butter (per day)	45

been a subject in the earlier vitamin studies which led to the initiation of the present investigation.

During the first portion of the study the subjects consumed a weighed mixed diet which was believed to meet the recommended dietary allowances in all respects (Food and Nutrition Board of the National Research Council, '48). The menu is listed in table 1. Quantitative collections of all excreta were made during the final 6 days of each unit of the study. Fecal losses for nitrogen, fat, crude fiber and calories were determined when the basal diet was eaten. During subsequent pe-

riods, test foods were introduced into the menu in amounts equal to two average adult servings per day. One-third of this quantity was consumed at each meal.

Determinations of the dry weight of the basal diet and of all test foods were made in advance so that the total dry weight of food consumed per 24 hours could be equalized during all metabolism periods. To achieve this, the weight of the basal diet was reduced, on the basis of the dry weight contributed by the test food, to such an extent that the total dry weight of food per day remained the same. These data are given in table 2.

TABLE 2

Food consumption during the 5 metabolism studies

PERIOD	TEST FOOD	PER CENT BASAL DIET
I	— —	100.0
II	Green frozen peas — 150 gm	92.2
III	Ice cream — 200 gm	82.4
IV	Almonds — 90 gm	80.6
V	Soybeans — 60 gm	88.6

Throughout the investigation the subjects conducted their usual work of teaching, research, or graduate study. They appeared free from illnesses at all times and were in good spirits.

The order of investigation of the various test foods was similar to that presented in the tables. In all cases, there was a lapse of at least 5 days between any two metabolism studies. All foods consumed during the entire investigation were purchased in advance, and were mixed carefully so as to be as uniform as possible. They were kept frozen until needed. Milk was obtained from a single lot; ice cream all came from one mix; bread was obtained from a single baking, etc.

Nitrogen determinations were made by the Kjeldahl-Gunning procedure. Crude fiber analyses were made by a modifica-

tion of the official A.O.A.C. method.[4] Heat of combustion values were obtained by ignition of the samples in a Parr bomb calorimeter. Total fat was determined following long-time extraction of the samples with ethyl ether. Carmine was used as the fecal marker.

RESULTS

Fecal losses per 24 hours for fat, nitrogen, calories and crude fiber were small when the subjects consumed the basal diet. The values did not vary greatly among the 4 subjects (table 3). While it appeared that the total general diet was fairly well digested, pieces of canned lima beans could be recognized in the feces from time to time and tomato seeds and small particles of wheat were identifiable. When 150 gm of a fancy grade of frozen peas were included in the menu per day, measurable increments in dry fecal weight were encountered, together with small increases in fecal nitrogen and calories. There was a much greater increase in the quantity of crude fiber present in the stools. The amount of fat contributed by the peas and the effect of ingesting this food upon fecal fat losses were negligible. The subjects regularly excreted particles of undigested peas.

The inclusion of 200 gm of vanilla ice cream in the daily menu resulted in minimum fecal losses of the nutrients studied. Particles of undigested food were seldom observed.

The consumption of 90 gm of toasted almonds, divided among the three meals of the day, resulted in a prompt and marked increase in fecal weight. Fragments of nut meats were obviously passing through the digestive tract and losses of nitrogen, fat, and calories in the feces were significantly increased. The ingestion of 60 gm of mature soybeans as baked beans likewise caused a definite increase in fecal dry weight. Fat and calorie losses were midway between those produced by the almonds and those by the basal diet alone. There was

[4] Crude fiber determinations were made under the supervision of Dr. Sybil Woodruff, Department of Home Economics, State University of Iowa, Iowa City.

some evidence of reduced utilization of the total nitrogen of the diet when this amount of soybeans was consumed, although individual differences in the response of the subjects with respect to nitrogen losses were large. Although intact soy-

TABLE 3

Daily excretion of nutrients in the stools during test periods

INTAKE		SUBJECT	DRY WEIGHT, FECES	FECAL LOSSES			
				Nitrogen	Fat	Crude fiber	Heat of combustion
	gm		*gm*	*gm*	*gm*	*gm*	*cal.*
Basal diet period							
Nitrogen	13.35	GB	14.4	0.88	1.3	1.41	70.4
Fat	119.9	IC	16.2	0.87	1.9	0.86	79.3
Calories	2466.4	AMR	14.1	0.82	2.0	0.56	66.0
Crude fiber	4.04	PMc	18.6	1.18	2.4	1.49	91.6
Basal diet	*92.2%*						
Green peas	*7.8%*						
Nitrogen	13.60	GB	18.7	1.08	1.8	3.29	89.9
Fat	113.6	IC	19.2	0.94	1.8	2.89	90.1
Calories	2460.6	AMR	16.0	0.96	1.9	1.34	74.1
Crude fiber	6.67	PMc	23.6	1.32	2.6	3.99	114.6
Basal diet	*82.4%*						
Ice cream	*17.6%*						
Nitrogen	12.41	GB	15.7	0.87	1.7	1.09	74.5
Fat	125.4	IC	12.8	0.82	1.4	0.72	64.5
Calories	2421.8	AMR	15.8	0.83	1.7	0.72	66.1
Crude fiber	3.25	PMc	14.0	0.94	1.5	0.89	70.1
Basal diet	*80.6%*						
Almonds	*19.4%*						
Nitrogen	13.99	GB	38.3	1.64	14.9	1.69	242.6
Fat	142.8	IC	30.0	1.32	11.3	1.53	194.4
Calories	2628.5	AMR	36.3	1.36	13.1	1.28	237.8
Crude fiber	5.95	PMc	38.7	1.60	15.1	1.61	242.3
Basal diet	*88.6%*						
Soybeans	*11.4%*						
Nitrogen	15.41	GB	22.9	1.18	4.2	1.57	121.8
Fat	117.2	IC	20.3	0.94	3.4	1.78	108.6
Calories	2534.3	AMR	18.5	0.96	3.9	0.62	100.1
Crude fiber	6.25	PMc	26.0	1.60	3.8	2.48	135.3

beans occasionally appeared to have passed through the digestive tract, small particles of the legume were more often observed.

Calculation of the proportion of a specific nutrient excreted in the feces which was due to incomplete digestion of the basal diet and that which was due to the ingestion of the test foods disclosed some interesting information regarding the relative utilization of the fat, nitrogen, and calories of the various foods. For example, 21 to 30% (depending upon the subject) of the calories furnished from almonds were accounted for in the feces. In the case of soybeans, from 14 to 21% of the calories were not absorbed. The data indicated nearly complete availability of the calories of ice cream. Due to the very small amount of energy furnished by the peas, it was impossible to estimate the per cent availability of calories from this food source with as high a degree of accuracy. Results relating to the digestion and absorption of fat from the 4 test foods revealed nearly complete absorption of fat from ice cream, less satisfactory use of fat from soybeans, and poorest digestion and absorption of the fat present in almonds. The green peas contributed too little fat for accurate comparison.

The digestion of the protein of the 4 foods, based on fecal losses of nitrogen, indicated that almonds provided the smallest percentage of digestible protein. There were small differences among the coefficients of digestibility of the protein of peas, soybeans, and ice cream.

DISCUSSION

It is apparent from the data presented in table 3 that the incorporation of moderate amounts of peas, baked soybeans, or toasted almonds into the daily menu produced a definite change in the composition of the stools of healthy young women, and resulted in increased losses of calories, fat, and protein from the body. These changes were beyond our expectations.

The addition of equally large servings of ice cream to the menu, however, produced no increase in loss of nutrients. In fact, the studies revealed excellent digestion of this food.

These findings are particularly interesting in view of our earlier observations concerned with the availability of riboflavin present in the same food sources. In the former vitamin experiments, 377 gm of ice cream were consumed by the subjects to furnish a test dose of 1 mg of riboflavin. At that time it was noted that the urinary excretion of riboflavin was equal to that produced when the basal diet was supplemented with 1 mg of the pure vitamin in the form of a water solution (Everson and associates, '48). Apparently the vitamin was readily available to the women subjects and the ice cream was completely digested.

While the quantities of almonds eaten were not identical in the two experiments (112 gm in the vitamin studies and 90 gm in the present investigation), both studies indicated some limitation in the use of nutrients furnished by this food source. Approximately 40% as much riboflavin was returned in the urine when almonds furnished the vitamin as when either pure riboflavin or ice cream supplied the factor. The present digestibility studies indicated that roughly 30% of the nitrogen, fat, and calories present in the almonds was excreted in the stools.

Due to the lower concentration of riboflavin in green peas, it was necessary that 714 gm of this vegetable be consumed to provide 1 mg of riboflavin. This large amount may have produced digestive disturbances not encountered in the more recent studies, when only 150 gm of this food were eaten, and therefore unusual losses of nutrients may have occurred. It should be emphasized, however, that while the ingestion of 150 gm of green peas appeared to change the composition of the stools to a smaller extent than was the case when either almonds or soybeans was the test food, there was a marked rise in the quantity of crude fiber present in the feces following the consumption of peas. It is believed that this increase was due mainly to the passage of many seed coats of the peas

through the digestive tract. The skins are a relatively rich source of riboflavin, although they contain minor amounts of protein, fat, and energy. Possibly this point alone explains the earlier observation that only approximately 40% as much riboflavin was excreted by the women subjects when green peas were the source of the vitamin as when the pure chemical or ice cream supplied the factor.

Similarly, it was necessary for the subjects to ingest excessive amounts of mature soybeans to secure 1 mg of riboflavin. In the earlier investigation, therefore, 250 gm of mature soybeans were eaten, approximately 4 times the amount consumed in the present studies. The return of the test doses of riboflavin furnished by soybeans which had been heated in different manners varied widely among the subjects (Baker, '47). In certain cases negligible amounts of the vitamin from the test food consumed could be accounted for in the urine. The maximum return of the vitamin was approximately one-half the amount excreted by the subjects when they ingested a water solution of pure riboflavin. The fact that fecal fat, nitrogen, and calories were increased to the extent shown in table 3 when only 60 gm of mature soybeans were consumed has led us to believe that incomplete digestion of the soybeans played an important part in limiting the quantity of riboflavin absorbed from this food source. While additional factors may be responsible for some of the differences observed in the availability of riboflavin in soybeans, almonds, green peas, or ice cream, it appears that incomplete digestion of the legumes and the nut meat may be an important factor in limiting the availability of this nutrient.

The present investigation shows the need for far more information than we have concerning the degree to which foods are digested. The results of these studies suggest too that there may be considerable overestimation of the value of certain foods as sources of calories, fat, and protein, as well as of the vitamins, if they are judged by their composition alone.

SUMMARY

Metabolism studies have been conducted on 4 young women to determine the degree to which green peas, ice cream, almonds, and soybeans are digested by this age group. Studies of these foods were initiated on the basis of earlier observations showing that a larger proportion of riboflavin furnished from ice cream was available for use than was available from two types of legumes or almonds.

The data revealed excellent digestion of ice cream and less satisfactory digestion of the other test foods. Losses of nutrients from certain foods were surprisingly large and apparently were due to incomplete digestion.

The incomplete digestion of certain foods is believed to influence not only the availability of protein, fat, and calories but also the availability of the vitamins present.

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THE SPECIFIC EFFECT OF NIACIN ON GROWTH [1]

BARNETT SURE, LESLIE EASTERLING AND SEQUAN JEU
Department of Agricultural Chemistry, University of Arkansas, Fayetteville

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In 1941 Sure and Dichek demonstrated that riboflavin produces a most pronounced effect on economy of food utilization, and that the increases in body gains are derived mainly from fats and to a lesser but appreciable extent from proteins. Recently Sure and Easterling ('49) found that pyridoxine also produces a marked effect on economy of food utilization, and that the increases in body gains are due largely to fat and protein and to a lesser but appreciable extent to increases of minerals in the animal body. Since in the studies cited the amount of food consumed by the vitamin-supplemented animals was the same as that ingested by the avitaminotic rats, the increased growth accompanied by increased economy of food utilization was due to the specific effect of these vitamins.

For years we have been attempting to produce niacin deficiency without any success, apparently due to the fact that the rat synthesizes this vitamin (Dann, '41). Krehl, Teply and Elvehjem ('45) reported a growth inhibition in rats, attributable to niacin deficiency, obtained by adding corn or corn grits to a purified diet very low in niacin. This growth retardation could be prevented by tryptophan as well as by niacin or by an increase in the level of casein. Spector and Mitchell ('46) found that the depression of growth on niacin-deficient diets is largely due to inadequate food intake, and

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that the marked growth following niacin addition is due chiefly to increased food consumption. Using our own biological technique in further experimental work in this area, it became apparent that niacin produces marked growth uncomplicated by the plane of nutrition.

EXPERIMENTAL

In the present communication the authors are reporting on the specific effect of niacin on the growth of the albino rat maintained by the paired-feeding procedure, using a technique which prevents bacterial synthesis of this vitamin. In order to elicit the optimum physiological effect of niacin, it is necessary to prevent bacterial synthesis of this vitamin. Employing the technique we developed it became evident that, even on a controlled food intake, niacin produces a marked specific effect on growth and on economy of food utilization. The following is the procedure we used: 10 pairs of male and 10 pairs of femals rats, 26 to 30 days old and weighing 45 to 58 gm each, were transferred from our stock colony diet and depleted of the vitamin B complex by subsisting for two weeks on the following purified ration: Casein,[2] 18; cellu flour, 2; Sure's salts No. 1 (Sure, '41), 4; corn oil,[3] 4; cod liver oil, 1; and cerelose, 71%. For the succeeding 12 days the animals were still deprived of niacin and they received the following ration throughout the experimental period: Casein,[4] 4; DL-methionine, 0.2; sulfathalidine, 2; corn oil, 3; corn meal (white), 40; Sure's salts No. 1, 4; vitamin A, D and E mixture in corn oil, 1; and cerelose, 45.8%. The purpose of the sulfonamide was to prevent bacterial synthesis of niacin. The vitamin A, D and E mixture used was that introduced by Schultze ('50).

This ration contains a low tryptophan content which is inadequate for niacin synthesis. When feeding of this diet

[2] Smaco. Extracted with 7 lots of hot 95% ethyl alcohol, 8 hours to each lot.
[3] Mazola.
[4] See footnote 2.

was begun, a litter mate of the same sex was restricted to the same food intake daily with the exception of Sundays. On Mondays the food intake was again balanced to that of the avitaminotic rat. When this ration was introduced, each animal began to receive daily, separate from the ration, 50 μg each of thiamine, riboflavin, and pyridoxine, 10 μg folic acid, 300 μg calcium pantothenate, 2 mg inositol, 6 mg *p*-aminobenzoic acid, and 9 mg choline chloride. After each animal in the various groups had been on this ration for 12 days, a litter mate in each paired group was given 100 μg niacin daily, separate from the ration, until the completion of the experiment (table 1, column 2).

On this dietary regime, and on the same food intake, pronounced increased gains in body weight because of niacin additions became apparent in about 30 days — in some cases a 50 to 60% increase. The symptoms of niacin deficiency were stunted growth, rough hair, alopecia, and occasional ophthalmia. No signs of dermatitis were evident such as are observed in human pellagra. The periods of depletion ranged from 35 to 88 days. The experiments were terminated when the avitaminotic animals showed, in addition to rough hair, alopecia, and eye lesions, prolonged maintenance at about the same body weight or stunted growth. Seventeen pairs of rats from the 20 groups used in this study were analyzed for protein, fat, moisture, and ash according to the technique previously described (Sure and Dichek, '41), in order to obtain information on the chemical composition of the body gains. In addition, 20 weaned rats, half of them male and half female, from the same rat colony, with weights and ages the same as those of the experimental animals taken for the niacin investigation, were analyzed for protein, fat and ash. The differences in the chemical composition of the dry carcasses of these rats and the dry carcasses of the animals sacrificed at the termination of the experiments represented the protein, fat, and ash contents of the body gains. The following is a summary of the results obtained; gains are represented by a plus (+) sign, and the figures represent averages for each

TABLE 1

The specific effect of niacin on growth

PAIR NO.	PERIOD OF VITAMIN DEPLETION	GAINS IN BODY WEIGHT	TOTAL FOOD INTAKE	GAINS IN BODY WEIGHT PER 100 GM FOOD INTAKE
	days	*gm*	*gm*	*gm*
1 – P ♂[1]	64	49	351.5	15.6
RC ♂		85	350.3	24.3
2 – P ♂	71	12	436.5	2.7
RC ♂		103	429.5	24.0
3 – P ♀	88	56	727.0	7.7
RC ♀		103	735.5	14.0
4 – P ♂	63	96	620.5	15.4
RC ♂		124	621.0	20.0
5 – P ♀	49	50	446.0	11.2
RC ♀		86	443.5	19.4
6 – P ♂	85	57	668.5	8.5
RC ♂		105	660.0	15.9
7 – P ♀	84	41	631.0	6.5
RC ♀		94	617.5	15.2
8 – P ♂	70	32	492.5	6.5
RC ♂		97	501.0	19.3
9 – P ♀	88	74	789.0	9.4
RC ♀		125	794.0	15.7
10 – P ♂	49	63	495.5	12.7
RC ♂		101	500.5	20.2
11 – P ♀	63	33	452.0	7.3
RC ♀		68	457.0	14.9
12 – P ♂	74	56	714.0	7.3
RC ♂		110	711.0	15.5
13 – P ♀	63	26	463.0	5.6
RC ♀		82	461.5	17.3
14 – P ♂	70	40	577.5	6.9
RC ♂		93	586.5	15.8
15 – P ♀	70	58	601.0	9.6
RC ♀		108	596.5	18.1
16 – P ♂	74	68	746.0	9.1
RC ♂		116	749.0	15.5
17 – P ♀	35	39	269.0	14.5
RC ♀		62	263.8	23.5
18 – P ♂	35	41	297.0	13.8
RC ♂		70	304.0	23.0
19 – P ♀	63	60	550.0	10.9
RC ♀		98	550.5	17.8
20 – P ♀	56	40	497.5	8.0
RC ♀		79	486.5	16.2
Average P		49.5	531.2	9.5
RC		95.5	540.9	18.3

[1] P — pathological — signifies niacin-deficient animal.
RC — restricted control — refers to the niacin-supplemented animal, which was restricted to the same amount of food as that consumed by the niacin-deficient rat.

animal. Niacin-deficient rats: fat, + 3.76 gm; protein, + 9.75 gm; ash (minerals), + 2.60 gm. Niacin-supplemented animals: fat, + 13.54 gm; protein, + 16.39 gm; ash, + 3.89 gm. It is apparent, then, that the greatest gains in body weight were from fat, followed by those from proteins and, to a lesser but significant extent, those from minerals.

On several occasions in the past the prevention of bacterial synthesis of niacin was attempted by introducing a sulfonamide into the ration, but after such procedure all the experimental animals began to decline in body weight. Recently it became apparent that the sulfa drug produces folic acid deficiency. The administration of 10 μg folic acid daily to each animal as a supplement to the synthetic vitamin B complex mixture made possible the inhibition of bacterial synthesis of niacin, so that the physiological effect following its addition, even on a controlled food intake, became pronounced.

In order to convince ourselves that we were dealing with an uncomplicated niacin deficiency, 4 avitaminotic rats, following a 6-week depletion period, were given 100 μg niacin daily. In the three weeks following such therapy the niacin-deficient animals gained about 50% in body weight.

SUMMARY

A modified procedure has been developed for the production of niacin deficiency per se in the albino rat, by incorporating 2% sulfathalidine in a low-casein, corn-containing ration supplemented with an adequate vitamin B complex mixture which furnishes 10 μg folic acid per animal per day. On such dietary regime, which prevents the bacterial synthesis of niacin, and after two weeks of vitamin B complex depletion and a subsequent 12 days of niacin depletion, paired feeding experiments were conducted on 10 pairs of male and 10 pairs of female rats, in which a litter mate of the same sex was given the same amount of food as that consumed by the niacin-deficient animal. The niacin-supplemented animals, which received 100 μg of this vitamin daily, in the course of 10 to 12 weeks gained an average of 93% more in body weight

than did the niacin-deficient rats, which we believe represents a *specific effect* of niacin addition.

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LYSINE DEFICIENCY IN RATS

II. STUDIES WITH AMINO ACID DIETS [1]

DAVID KLIGLER AND WILLARD A. KREHL

Yale Nutrition Laboratory, Department of Physiological Chemistry, Yale University School of Medicine, New Haven, Connecticut

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INTRODUCTION

In a previous paper (Kligler and Krehl, '50) the present authors reported on the difficulty of creating an uncomplicated lysine deficiency in young rats when zein was used as the dietary protein. The principal reason for this was determined to be the poor digestibility of the zein, and an explanation was offered in terms of the concept of amino acid imbalance. Even when supplemented with amino acids to provide the minimum daily requirements of all those essential for growth, zein diets were associated with only very small increments of weight. Attempts at further amino acid supplementation of this corn protein resulted in some improvement of the growth rate which, however, never equalled the optimum rates obtained with casein diets. The use in lysine deficiency studies of hydrolysates of zein, of "deaminated" casein,[2] and of gliadin as the sources of dietary amino nitrogen has similarly introduced various complicating experimental factors (Winternitz, '42; Borchers et al., '42; Harris et al., '43; Holt and Albanese, '44; Geiger and Hag-

[1] The authors are indebted to the National Vitamin Foundation, New York, N. Y., and to the James Hudson Brown Memorial Fund of the Yale University School of Medicine for grants in support of this work.

[2] "Deaminated" casein in casein treated with nitrous acid, eliminating about 50% of the free amino groups, and hence about 50% of the lysine (Holt and Albanese, '44).

erty, '49). Whether the signs observed in lysine-deficient zein-fed animals, therefore, are those of a specific deficiency state or the result of other shortcomings of the diet can best be determined by the use of mixtures of pure amino acids.

The problem of finding such an amino acid mixture, which will support high rates of growth in young animals over a relatively long period of time, has only recently been solved by the addition of varying amounts of the non-essential amino acids to mixtures containing the 9 essential ones (Rose et al., '48; Maddy and Elvehjem, '49; Ramasarma et al., '49). With the source of dietary amino nitrogen thus controlled, it becomes possible to study chronic deficiency states produced by the removal from the diet of single essential amino acids.

In addition to reporting on the lysine-deficient young animal thus developed, and comparing it with the zein-fed deficient animal, the current paper presents some additional findings concerning zein as a source of dietary nitrogen for the rat.

EXPERIMENTAL

Animals

Sprague-Dawley weanling male rats aged 4 weeks, with weights within a range of 35 to 45 gm, were housed in individual, screen-bottom cages in a room with both temperature (25 to 26°C.) and relative humidity (50 to 55%) controlled. The animals were given food and water ad libitum, and were weighed at least once a week.

Diets

The composition of the diets used in this investigation is given in table 1. The amino acid composition of the casein and zein diets is compared, in table 2, with that of the pure amino acid diets, and with the minimum requirements of the essential amino acids as given by Rose ('37). It can be seen that, theoretically at least, all the diets provide these minimum requirements. The amino acid mixtures were prepared in the proportions used by Ramasarma and associates ('49),

TABLE 1

Composition of diets

COMPONENTS	DIETS			
	A	B	P–2	P–3
	%	%	%	%
Basic constituents [1]	24.1	24.1	29.10	29.10
Dextrin [2]	50.7 [3]	49.2	50.49	51.73
Casein [4]	25.0 [3]			
Zein [5]		25.0		
AAM–1 [6]			7.85	7.85
AAM–2 [6]			9.69	9.69
L-cystine	0.2	0.2		
DL-tryptophan		0.3		
DL-histidine·HCl·H_2O		0.2		
L-lysine·HCl		1.0	1.24	
Na-glutamate [7]	(1.0)		1.00	1.00
NaH_2PO_4			0.63	0.63

[1] Lard (Swift'nin) 10.0%; vitaminized corn oil (Mazola) 5.0%; salts IV 4.0%; choline chloride 0.1%; vitaminized dextrin 5.0% (in diets A and B), 10% in diets P–2 and P–3; total 24.1% or 29.1%.

Vitaminized corn oil was prepared so that, at a level of 5.0% in the diet, the fat-soluble vitamins would be available in the following amounts per 100 gm of diet: 3,000 U.S.P. units vitamin A; 430 U.S.P. units vitamin D; 12.5 mg α-tocopherol acetate.

Salts IV (Phillips and Hart, '35; Hegsted et al., '41) was modified to contain cobalt ($CoCl_2 \cdot 6H_2O$, 0.6 gm).

Vitaminized dextrin was prepared so that at a level of 5% in the diet the water-soluble vitamins would be available in the following amounts per kilogram of diet: 5 mg each of B_1, B_2, B_6; 50 mg each of niacin and pantothenic acid; 0.5 mg each of biotin and folic acid; 100.0 mg each of inositol and para-aminobenzoic acid; 5.0 mg of menadione.

[2] Amijel (Corn Products Refining Co., New York, N. Y.).

[3] In diet AP, used as control diet for diets P–2 and P–3, the proportions of casein and dextrin were 19% and 55.7%, respectively.

[4] Labco devitaminized casein (Borden).

[5] Corn Products Refining Co.

[6] See table 2.

[7] "Accent." International Minerals and Chemical Corp., Chicago, Ill. Added as a supplement to mixture indicated in above items: 1.0 gm added to 100 gm of the mixture (indicated by parentheses).

TABLE 2

Amino acid composition of diets and of reference casein and zein

AMINO ACIDS	IN REFERENCE CASEIN [1]	IN 19% CASEIN DIET (AP)[2]	IN REFERENCE ZEIN [1]	IN 25% ZEIN DIET (B)[3]	IN AMINO ACID DIET [4] (P–2)	MINIMUM REQUIREMENT, GROWING RAT [5]
	%	% of diet	%	% of diet	% of diet	% of diet
Non-essential						
Glycine	0.5	0.1	0.0	0.0	0.90	
Serine	6–7	1.1 +	?[6]	?	0.64	
Tyrosine	6.4 ± 0.4	1.2	5.0 ± 1.2	1.2	1.79	
Cystine	0.36	0.1 (0.3)[7]	0.8 ± 0.1	0.2 (0.4)[8]	0.26	
Asparagine	6.3	1.2	3.4	0.8	1.56	
Alanine	5.6	1.1	9.9	2.5	1.54	
Proline	8.2	1.6	9–12	2.2 +	1.16	
Hydroxyproline	2	0.4	1.0	0.2	none	
Glutamic acid	22.8	4.3	35.6	8.9	2.00	
Essential						
Tryptophan	1.8 ± 0.2	0.3	0.1	(0.3)	0.20	DL 0.2
Histidine	2.5 ± 0.3	0.5	0.9 ± 0.2	0.2 (0.4)	0.54 (0.50)[9]	DL 0.4
Phenylalanine	5.2 ± 0.5	1.0	6.4 ± 0.7	1.6	0.90	DL 0.9
Leucine	12.1	2.3	23.7 ± 2.1	5.9	0.80	L 0.8
Isoleucine	6.5	1.2	4.3 ± 0.4	1.1	1.00 (0.50)	L 0.5
Threonine	3.9 ± 0.1	0.7	2.4	0.6	1.00 (0.50)	L 0.5
Methionine	3.5 ± 0.3	0.7	2.4	0.6	0.60	DL 0.6
Valine	7.0	1.3	2.4 ± 0.9	0.6	1.40 (0.70)	L 0.7
Arginine	4.1 ± 0.2	0.8	1.6 ± 0.2	0.4	1.25 (1.20)	L 0.2
Lysine	6.9 ± 0.7	1.3	0.0	(1.0)	1.24 (1.00)	L 1.0

[1] From Block and Bolling ('45), p. 304.

[2] See table 1. Amino acid composition of 25% casein diet is not given, since obviously it provides even higher levels of essential and non-essential amino acids than does the 19% casein ration.

[3] See table 1.

[4] See table 1. Diet P–3 contains the same levels of amino acids, without lysine. AAM–1 (total: 7.85%). AAM–2 (amino acid mixture 2) contains all the essential amino acids except lysine, plus glutamic acid (total: 9.62%).

[5] From Rose ('37). Letters indicate the physiologically active forms of the essential amino acids.

[6] No analysis for serine in zein is available.

[7] Parentheses enclose total level when supplement of L-cystine is included.

[8] Figures in parentheses indicate total levels when supplements of pure amino acids are added.

[9] Figures in parentheses in this column indicate levels of physiologically active forms of the essential amino acids in the mixture, where DL forms or hydrochlorides were used.

who were able to produce growth rates in young rats nearly as rapid as those produced with isonitrogenous casein rations. The proportions of the non-essential amino acids were chosen arbitrarily, while the essential amino acids were supplied according to the minimum requirements established by Rose and co-workers (Rose, '37; Rose and Womack, '46). The high levels of arginine and glutamic acid were selected empirically, on the basis of observed improvement in growth responses. The amino acid mixtures were incorporated in diet P-2 and, with the exclusion of lysine, in diet P-3. NaH_2PO_4 was added to supply the phosphorus contained in an 18% casein diet, for which the salts IV mixture was devised. Monosodium glutamate was added, in line with the observation (Smith and Elvehjem, '50) that the initial lag in growth observed when young rats and mice are placed on amino acid rations is overcome when this compound is used.

A low-nitrogen ration was prepared containing one-half the amounts of each of the two amino acid mixtures present in diet P-2, or 3.93% AAM-1 and 4.85% AAM-2, and thus providing only half the minimum daily requirements of the essential amino acids, including lysine, of which 0.5% was supplied. Total dietary nitrogen was thus 9.42%, as against 18.78% in diet P-2. Except for an increased amount of carbohydrate, the basic dietary constituents were otherwise unchanged.

Methods

In general, weight gain was used as the principal criterion of growth. The growth rate of young male rats on a 19% or 25% casein diet was used as the indicator of optimum weight gain in these experiments (table 3). Additional studies included, in the case of the lysine-deficient animals, the preparation of histological sections and, in the case of animals given the supplemented zein diet, the determination of nitrogen balance.

The nitrogen balance study was carried out as follows. During the 6-day collection periods the animals were placed

* Figures in parentheses in this column indicate levels of physiologically active forms of the essential amino acids in the mixture, where DL forms or hydrochlorides were used.

in screen-bottom cages supported over metal funnels on a specially constructed rack. Water and food were allowed ad libitum. In the preparation of the feces for analysis, each total 48-hour collection was dried, freed of obviously foreign material, weighed, and pulverized. A 0.1-gm sample was taken for micro-Kjeldahl determination, after predigestion with 20 ml of 20% H_2SO_4 for one hour in an autoclave. The analysis of food, urine and feces was carried out, with minor

TABLE 3

Growth responses to control and experimental diets

DIET [1]	NO. OF DAYS ON DIET	NO. OF ANIMALS	AVERAGE GAIN/ ANIMAL/DAY	AVERAGE DAILY RANGE OF GAINS
			gm/day	*gm/day*
25% Casein (A)			5.6–5.8	
19% Casein (AP)			5.6	
25% Zein (B)			0.8	
Amino acid diet with lysine (P–2)	14	4	4.4	3.7–4.7
	30	4	4.2	3.4–4.9
	60	4	4.0	3.8–4.5
Amino acid diet without lysine (P–3)	50	19	— 0.1	
	80	3	— 0.1	
P–2 after P–3	35 after 50	4	4.0	3.9–4.1
A after P–3	35 after 50	4	6.2	5.5–6.5
Low-N diet	30	4	— 0.5	

[1] See table 1.

modifications, according to the method described by Ma and Zauzaga ('42). Standardization of the procedure was carried out before each series of determinations with the use of known solutions of $(NH_4)_2SO_4$, and the steps of digestion, distillation, and titration were found to be accurate within 1 to 2%. A larger error was inevitably introduced due to the crudity of the collection apparatus, for which approximate corrections have been made. Experimental determination of the nitrogen in the casein and zein diets gave the basis for calculation of the average daily nitrogen intakes.

RESULTS

Amino acid diets

The growth of weanling male rats fed the 19% casein diet in this study averaged 5.6 gm/animal/day over a 40-day period, a rate higher than that usually regarded as optimum in growth experiments for the strain of rat used. The average rate of growth of animals fed the complete amino acid ration (diet P-2) was 4.2 gm/animal/day, only 75% of the optimum rate.[3] This rate is nevertheless a good one, sustained over an adequate period of time, and it is reasonable to assume that the removal of one of the essential amino acids from the mixture would produce a relatively specific deficiency.

Weanling male rats placed on the lysine-deficient amino acid diet (diet P-3) began to show obvious signs of deficiency after three to 4 weeks of feeding. All these animals failed to grow, and instead lost, over the first 50 days, between one-seventh and one-ninth of their initial body weights, which had been 35 to 45 gm. Of the 20 rats started on diet P-3, one died at the end of one week, while the remainder were still alive after 50 days. Of 6 animals continued on the deficient diet for a longer period, three survived 80 days but only one was still relatively hardy at that time.

The signs of deficiency were a ruffled, mangy coat, a hunched stance, and an unsteady gait. The animals were emaciated, but remained restless and active. Before death the teeth were blackened, gum hemorrhages appeared, and there was diarrhea. Growth of the long bones and of the viscera appeared to have been maintained to some extent. Grossly the tissues of the 80- and 120-day-old rats appeared denuded of fat, and the viscera and endocrine organs were relatively large, as might be expected in a very young animal, but the appearance was otherwise unremarkable. Histological sections of the

[3] Although the amino acid constitution was the same, tue diets here used differed from those of Ramasarma et al. ('49) in that the latter incorporated only 5% fat and used sucrose as the carbohydrate, whereas in the current study 15% fat and dextrin were used.

liver, heart, kidneys, adrenals, and pituitaries showed no significant evidence of pathology, save for the expected depletion of liver fat.

Two adult animals placed on diet P-3 for a 90-day period lost 83 and 81 gm, respectively, an average of one-fifth of their initial body weights of 399 and 396 gm. Two control animals of comparable age and weight, fed the complete amino acid ration (diet P-2), did not make substantial gains, but did successfully maintain their body weights.

Four male rats placed on diet P-2 after 50 days on diet P-3 were able to grow at a rate comparable to that observed in animals fed diet P-2 from the outset, and all signs of deficiency disappeared. The response of 4 animals placed on diet A (25% casein) after 50 days on diet P-3 was similarly excellent. The potential for growth on a good diet was not impaired by the fairly long period of deficiency.

Four male weanling rats placed on the inadequate low-nitrogen diet lost about 15 gm in 4 weeks, or one-third of their initial body weights, with the appearance, after three to 4 weeks, of deficiency signs and pathological findings similar to those observed above under conditions of specific lysine deficiency.

Parenteral injections of preparations of growth hormone into lysine-deficient and protein-depleted animals produced no growth response.

Zein diets

In the experiments previously reported (Kligler and Krehl, '50) it was found that the theoretically adequate zein diet (diet B), in itself unsatisfactory, supported good growth when 5% of casein was added, and supported fair growth when an amino acid supplement of arginine, valine, and threonine was added. Enzymatic hydrolysis of the zein, a difficult procedure *in vitro*, did not lead to an improvement in the growth response, even though the necessary amino acids were added, as to diet B, in order to provide the minimum requirements.

TABLE 4

Results of nitrogen balance determinations

DIET	ANIMALS [1]	AVE. BODY WEIGHT	AVE. DAILY WEIGHT GAIN	AVE. DAILY FOOD INTAKE	(a) N_2 INTAKE/ GM BODY WEIGHT [2]	(b) FECAL N_2/ GM BODY WEIGHT	(c) URINE N_2/ GM BODY WEIGHT	N_2 BALANCE = a−(b + c)
		gm	*gm*	*gm*	*mg*	*mg*	*mg*	*mg*
A	I [3] (3)	94.3	7.6	9.0	3.02	0.08	0.37	2.57
	II (3)	270.6	4.6	12.6	1.48	0.09	0.38	1.00
B	III (4)	85.4	— 0.3	6.0	2.41	0.99	0.32	1.10
	IV (8)	84.0	0.4	5.1	2.11	1.12	0.25	0.74
	V (3)	87.8	— 0.1	5.1	1.97	0.90	0.27	0.80
	VI (4)	380.9	0.0	17.2	1.57	1.24	0.38	— 0.05
B-2 [4]	VII (3)	178.9	4.0	10.1	1.93	1.14	0.48	0.31

[1] The numbers in parentheses indicate number of rats used.

[2] *Animals:* Group I — weanling males after 10 days on diet A, chosen at a time when their weights roughly approximated those of groups III, IV, and V.

Group II — young adult males fed diet A for one month (starting weights 49–51 gm), which were of the same age as group III.

Group III — 60-day-old animals fed diet B for one month (starting weights 48–54 gm).

Group IV — 90-day-old animals fed diet B for two months (starting weights 38–42 gm).

Group V — 90-day-old animals fed diet B for two months (starting weights 43–46 gm).

Group VI — Adult animals fed diet B for one month.

Group VII — 90-day-old animals fed diet B-2 for one month, belonging to the same series as groups II and III (starting weights 49–52 gm).

[3] The formula for this calculation is $K \times \frac{\text{ave. daily food intake}}{\text{ave. body weight}}$, where K is the determined dietary value of N_2 (mg/gm diet). (Diet A, K = 31.5; diet B, K = 34.2.)

[4] Diet B-2 was diet B with a supplement of arginine, threonine and valine.

Two further findings related to the problem of the nutritive value of zein emerged from the nitrogen balance studies. The first was the presence in the feces of zein-fed animals of 10 times or more the amount of nitrogen present in the feces of casein-fed animals of similar age or size (table 4: compare in column b groups I and II with group III). This appears to be fairly clear-cut evidence for the inefficient digestion of zein by the rat, growing or adult (group VI). The large fecal nitrogen output was present also in animals fed the zein rations improved by the addition of the three-amino acid supplement (group VII).

The second finding of interest was the consistently positive nitrogen balance of zein-fed animals, which apparently persists in these animals despite negligible gains in weight and large fecal nitrogen output. The average difference between intake and output, as related to body weight, for all three groups of zein-fed young animals growing less than 1 gm/day (groups III, IV, V), when weighted for the number of animals in each group, was 0.85 mg nitrogen retained per gram body weight per day. This is compared with an average of 1 mg nitrogen/gm body weight/day retained in rapidly growing casein-fed animals gaining 4.2 to 5.0 gm/day, and 2.6 mg/gm in casein-fed animals growing 7.2 to 8.1 gm/day (groups II and I, respectively). Group VII, on the other hand, which was gaining fairly rapidly on the improved zein diet, was nevertheless not in strongly positive balance, but this latter result was not confirmed by repeated determinations.

DISCUSSION

As a result of the development of pure amino acid rations which promote good growth in young rats over a long period of time, it is now possible to prepare a diet from which single essential amino acids can be excluded. With this excellent experimental tool, one can study the effects of the complete absence of lysine from the dietaries of growing and adult animals. In terms of gross and microscopic evidences of deficiency, the amino acid-fed, lysine-deficient young rat ap-

peared, in the current study, no different from its zein-fed, lysine-deficient counterpart studied earlier (Kligler and Krehl, '50). There were, however, some differences in the life histories of the deficiency disease, in that obvious signs of deficiency appeared in amino acid-fed young rats an average of one week later than in zein-fed animals, while the latter group survived nearly twice as long as the former in the absence of dietary lysine. Animals from both groups were able to grow well, after a period of deficiency, when they were placed on a casein diet, indicating that the potential for growth was not impaired. It was confirmed, therefore, by means of the lysine-deficient, amino acid diet, that the absence of lysine does indeed prevent normal growth, while permitting limited visceral and skeletal development at the apparent expense of fat and connective-tissue elements.

The depletion of fat deposits, despite an adequate caloric intake, would tend to support the impression (Winternitz, '42) that lysine-deficient animals require more energy than usual for the maintenance of even a negative nitrogen equilibrium. An alternative suggestion is that the hypoproteinemia in these animals (Harris et al., '43) is part of a state of general tissue depletion or starvation, and that the absence of depot fat simply corroborates this. The similarity between the picture of lysine deficiency described above and that of protein depletion in general is evident from the work of Winters et al. ('27), who found that after 40 days on a low-protein diet young rats showed persistent skeletal growth, increase in visceral weights, and diminished fat deposition and muscle development. These results were confirmed in the current study with the use of the amino acid diet in which all the essential amino acids were present, but at half their minimum requirements. The animals thus depleted did not differ grossly or microscopically from their contemporaries deficient only in lysine. None of these animals responded by weight gain to injections of growth hormone, but all responded rapidly when placed on a good diet. It is suggested, therefore, that lysine deficiency exerts a pathological effect

similar to that of severe states of protein depletion or starvation, the animal responding by using up his fat and carbohydrate reserves.

While this investigation was concerned principally with the growing rat, findings have also been recorded suggesting an essential role for lysine in the nutrition of adult rats. Fully grown animals placed on the lysine-deficient, amino acid diet lost considerable weight, lending support to one view expressed in the literature, namely that small amounts of lysine are essential for the maintenance of the adult rat (Neuberger and Webster, '45; Wissler et al., '48). This is contrary to the findings of Mitchell ('47).

The presence of large amounts of nitrogen in the feces of zein-fed animals, in the nitrogen balance studies, lends further support to the well-established observation that zein is poorly digested *in vivo* as well as *in vitro* (Mason and Palmer, '35; Albanese et al., '49; Geiger and Hagerty, '49; Kligler and Krehl, '50). More difficult to explain is the observation that animals growing very poorly on a zein ration (fortified with essential amino acids to provide theoretically adequate levels) are nevertheless in positive nitrogen balance. Gortner ('50) has recorded similar observations. If the assumption is made that an experimental error, previously unaccounted for, is at fault, then corrections would have to be introduced in all groups of data. The result would be a lower positive balance not only for the slowly-growing, zein-fed animals, but for the rapidly-growing, casein-fed controls. It is felt, therefore, that the result is not a false one, and must somehow be correlated with other findings related to zein digestion and utilization.

SUMMARY

1. The current availability of an amino acid ration, containing both essential and non-essential amino acids, which will support high rates of growth over long periods of time in young rats, has made possible the study of specific single amino acid deficiencies, uncomplicated by factors introduced

through the use of proteins which are inadequate as sources of nitrogen.

2. The picture of lysine deficiency thus created was found not to differ perceptibly from that of lysine deficiency induced by the use of the protein, zein. In addition, this deficiency state was found to be very similar to that induced under circumstances of general protein depletion, with the use of an amino acid ration containing inadequate amounts of all the essential amino acids as well as the non-essential amino acids.

3. Adult rats fed the amino acid diet were found to require small amounts of lysine for the maintenance of body weight.

4. Additional data were gathered in nitrogen balance studies to support previous observations that zein is poorly digested by the growing rat, with large amounts of nitrogen being lost in the feces. An anomalous finding in these studies was the presence of a positive nitrogen balance in animals growing very poorly on the amino acid-fortified zein ration.

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NIACIN DEFICIENCY IN THE RHESUS MONKEY[1]

D. V. TAPPAN, U. J. LEWIS, U. D. REGISTER
AND C. A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

TWO FIGURES

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Although previous workers have succeeded in demonstrating conditions in monkeys resembling a niacin deficiency when they fed natural diets, particularly those high in corn, more recent attempts to produce such a deficiency condition with purified diets have proved inconclusive, since the animals have failed to respond to niacin therapy.

Chick and Hume ('20) produced a condition in monkeys closely resembling human pellagra by feeding a natural diet presumably adequate for the nutrition of this species but low in protein, with corn supplying most of the protein. Supplements of crude amino acids including tryptophan proved to be of little or no value in correcting the condition. Significant improvement was brought about only when the animals were returned to a "normal" diet of rice, wheat germ, autoclaved milk and cabbage.

Harris ('37) produced a syndrome in monkeys characterized by loss of appetite, diarrhea, vomiting, denudation, and scaly hands and feet by feeding a diet containing about 72% corn meal and deficient in the human antipellagra or canine antiblacktongue factor. The animals receiving this diet could

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be protected with supplements of liver extract powder, yeast, or by a substitution of whole wheat for the maize in the diet. With the demonstration by Elvehjem et al. ('37) that nicotinic acid is identical with the antiblacktongue factor, Harris ('38) succeeded in showing that monkeys made deficient through consumption of the "blacktongue" ration (Goldberger and Wheeler, '28) responded to niacin therapy. The work of Day et al. ('38) using partially purified diets, however, indicated that a deficiency in the factor which they termed "vitamin M" might also have been present in the animals used by Harris. In later studies, Day et al. ('40) used a modified Goldberger diet very similar to that of Harris and failed to demonstrate a successful response with supplements of nicotinic acid, riboflavin, thiamine and ascorbic acid.

Although various species have been shown to need niacin when fed purified rations (Schaefer et al., '42; Briggs et al., '42; Wooley and Sebrell ,'45), the attempts by Cooperman, McCall, Ruegamer and Elvehjem ('46b) to demonstrate a need for niacin by monkeys receiving purified or partially purified rations were not successful because of the concurrent production of another deficiency. These workers fed a 40% corn ration to monkeys as a possible means of producing a niacin deficiency, since rats had been shown (Krehl et al., '45a, b) to require supplements of niacin or tryptophan when receiving such a diet. The consumption by the monkeys of either the 40% corn ration or an 18% casein ration led to eventual weight loss and reduction in hemoglobin values. In the latter case the deficiency may undoubtedly be partly attributed to the low level of folic acid administered. Neither niacin nor tryptophan was capable of reversing the conditions, while whole liver or other supplements high in the monkey anti-anemia factor brought about rapid improvement. The deficiency resulting from the consumption of these rations differed from deficiencies of riboflavin (Cooperman et al., '45), folic acid (Cooperman et al., '46a) or pantothenic acid and vitamin B_6 (McCall et al., '46), which also required supplements of the anti-anemia factor for correction, in that

no initial response occurred with niacin as did occur with the other vitamins.

The present study has been directed toward the production of a niacin deficiency in monkeys through the use of a purified diet containing 9% casein. The resulting deficiency has been corrected by the administration of either niacin or tryptophan.

EXPERIMENTAL AND RESULTS

The animals were housed in individual metal cages in an air conditioned animal room, as described previously by Waisman et al. ('43). A ration consisting of 9% casein,[2] 82% sucrose, 4% salts IV (Hegsted et al., '41) and 5% corn oil supplemented with 9 mg menadione and approximately 5,000 U.S.P. units vitamin A and 500 U.S.P. units vitamin D (as oleum percomorphum[3]) per kilogram was given to the animals twice daily in 100- to 200-gm amounts. Other vitamin supplements were given three times weekly as described earlier (Waisman et al., '43) in amounts sufficient to provide 1 mg thiamine hydrochloride, 1 mg riboflavin, 1 mg pyridoxine hydrochloride, 3 mg calcium pantothenate, 25 mg choline chloride, 25 mg *p*-aminobenzoic acid, 25 mg *i*-inositol, 0.02 mg biotin, 0.5 mg folic acid and 25 mg ascorbic acid daily. Niacin or tryptophan supplements were given with the regular vitamin mixture when these were administered.

In a preliminary experiment to determine the ability of this diet to support adequate growth in monkeys when supplemented with niacin, a male animal approximately one year of age that had previously received an 18% casein diet with complete vitamin supplements was given the above-described regimen. Niacin supplements were administered as indicated in figure 1. Since the animal had been gaining optimally on the pre-experimental diet, the decline in weight after approximately three months on the 9% casein ration may be attributed to a deficiency in the experimental diet. The sharp rise in weight beginning after 5½ months on the experiment,

[2] Vitamin-test casein, General Biochemicals, Inc.
[3] Mead, Johnson and Co.

as indicated on the graph, was accompanied by an edema apparent primarily around the face and scrotal area. A 5-mg dose of niacin brought about loss of the excess body fluid, and the continued decline in weight was arrested by one 10-mg dose of niacin. Approximately 10 mg of niacin per week were necessary to produce a steady weight increase, while 35 mg weekly brought about a rapid and continuous gain.

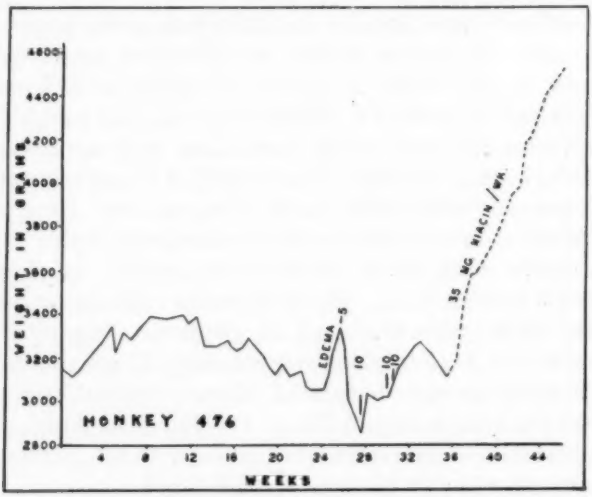

Fig. 1 Effect of supplementation of 9% casein diet with niacin. Figures represent individual doses of niacin, in milligrams.

Five young female monkeys (numbers 517, 525, 526, 527 and 528) with initial weights of 1,400 to 2,200 gm were then placed on the low protein-niacin deficient basal diet for periods of from three to 9 months prior to the administration of supplements. In no case was supplementation initiated until depletion was indicated by a weight plateau of at least three months' duration, or by a loss in weight of 300 gm or more. Only two of these animals will be discussed in some detail, since the responses obtained were typical of the entire group.

Animal 528 after 4 months on the depletion ration responded to a 10-mg dose of niacin with a sharp increase in weight, as shown in figure 2, and no other supplement was necessary for a period of approximately 16 weeks. When a significant weight loss had occurred, a series of doses of 500

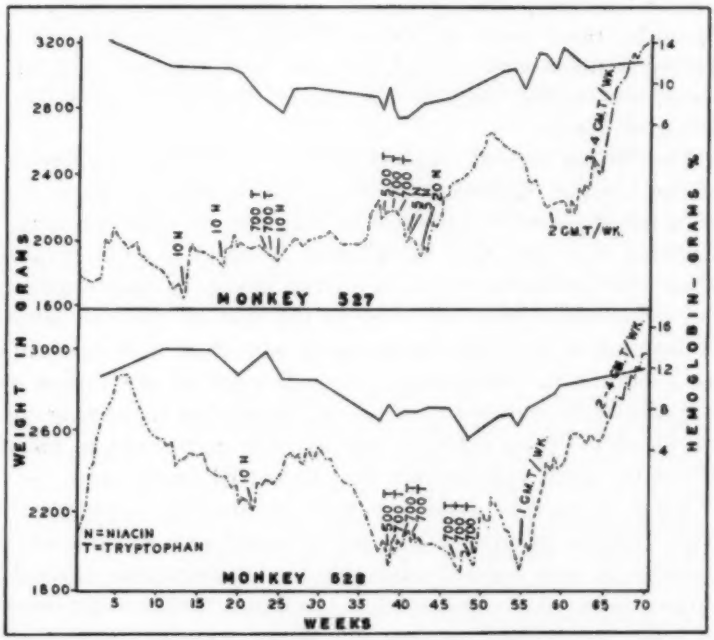

Fig. 2 Response of monkeys receiving 9% casein diet to niacin and tryptophan supplementation. Solid lines show hemoglobin concentrations, while broken lines indicate weight values. Figures represent individual doses, in milligrams, of niacin or tryptophan.

to 700 mg each of DL-tryptophan successfully maintained the weight of the animal when administered at approximately weekly intervals, or promoted slight gains in weight when administered more frequently. Weight losses occurred regularly when the supplements were temporarily discontinued.

In contrast to the above animal, monkey 527 required a shorter depletion period and responded to niacin supplements with only small weight increases. Between the 11th and 24th weeks of the experiment three 10-mg doses of niacin and two 700-mg doses of DL-tryptophan were given to this monkey in order to keep the weight at a fairly constant level. Loss in weight beginning during the 9th month was temporarily reduced by three doses of 500 to 700 mg of tryptophan given during a three-week period, but the sharp decline that followed was halted only by the administration of a total of 30 mg of niacin.

The 700-mg dose of tryptophan was selected as an estimate of the amount equivalent to 10 mg niacin but, of course, no data for the conversion ratio in this species were available. Krehl et al. ('46b) have shown that 50 mg of L- or DL-tryptophan are equivalent to 1.0 or 1.5 mg niacin in counteracting the deleterious effect of corn in the diet of the rat, while Henderson et al. ('49), in studying quinolinic acid excretion as a method for determining the efficiency of conversion of tryptophan to precursors of niacin, found that the conversion ratios varied from 0.2% in man and the guinea pig to about 1% in the lamb, calf and pig, and 10 to 20% in the rat.

Since it seemed apparent that tryptophan supplements were capable of preventing loss of weight in at least some animals, it was deemed advisable to determine the effect of larger doses. As is indicated in the figure, with weekly doses of 1 gm tryptophan monkey 528 began a steady increase in weight which was maintained continuously until the end of the experiment. The administration of 2 to 4 gm per week during the final 6-week period resulted in no more rapid growth than the 1 gm level. When 2 gm of tryptophan per week were administered to monkey 527, a slight but very unsteady gain in weight occurred. With an increase in the supplementation level to 3 to 4 gm per week, however, a continuous weight increase was also maintained by tryptophan in this animal.

Hemoglobin values for the monkeys were determined at frequent intervals throughout the course of the experiment and were measured weekly during critical stages of the work. The hemoglobin concentration of monkey 476, not subjected to a prolonged depletion period, was 14.0 gm % at the beginning of the experiment and remained within a range of ± 2.3 gm % of this value.

The hemoglobin charts for monkeys 527 and 528, included in figure 2, show values fairly typical of the remaining animals. The average hemoglobin concentration for these monkeys at the end of the experimental supplementation period was 13.8, while 4 of the 5 animals showed values of 5.4 to 7.8 gm % during the stages of most severe depletion.

No reversals of neutrophil-lymphocyte ratios were observed during this work, indicating that the regimen used produced no deficiency in the monkey anti-anemia factor.

DISCUSSION

Since monkey 476 and monkey 525 maintained optimum growth rates while receiving niacin, it is apparent that the diet used was adequate except for its niacin content. Monkey 525 required 9 months to show a deficiency in niacin as indicated by a loss in weight, and then grew satisfactorily upon receiving an average of 6 mg niacin per week. While monkey 528 gained weight for a period of about 8 weeks after receiving only 10 mg of niacin, somewhat higher requirements were exhibited by other animals. Although an absolute value for the requirement of this species for niacin could not be determined with the small number of animals used, 10 mg per week are apparently quite adequate for maintaining optimum growth in monkeys subjected to these experimental conditions.

As with niacin, the tryptophan requirement varied over a considerable range. Monkey 528 grew steadily upon the administration of 1 gm tryptophan per week, while 2 gm per week were sufficient for maintenance of weight but supported only very irregular growth in monkey 527. In the case of

this animal it was necessary to administer 3 to 4 gm of tryptophan per week for growth to be optimum. The other two animals maintained on tryptophan for extended periods were found to require 1 to 2 gm per week for satisfactory growth.

Although only approximate values for the relative requirements for niacin and tryptophan have been established by these studies, the similar growth responses obtained with weekly supplements of 6 to 35 mg niacin and 1 to 4 gm DL-tryptophan indicate a very inefficient conversion of tryptophan to niacin in monkeys. No attempt was made during the course of these studies to determine the relative effectiveness of the two isomers of tryptophan.

The only deficiency symptoms other than weight loss and significant reduction in the hemoglobin content of the blood evident in any of the animals throughout the entire study were the edema observed in monkey 476, accompanied by a darkening of the fingers extending to the second joint, and a mild dermatitis manifest as small red blotches over the face and other exposed areas of the skin about 4 weeks later when a steady gain in weight had been resumed. The condition was successfully corrected by the continued administration of the niacin supplements.

During a later stage of these studies two monkeys receiving regular tryptophan supplements and another receiving niacin supplements were given both tryptophan and niacin, each in amounts sufficient for supporting growth, for a period of three weeks. Neither during the time of the combined supplementation nor after the resumption of single supplements were increases in growth rate noted in any of the animals. It is tentatively postulated, then, that niacin does not enhance the utilization of tryptophan in the monkey as has been demonstrated in the rat by Krehl et al. ('46a).

The findings reported here are essentially in agreement with those of Cartwright et al. ('48), in which swine maintained on a low-protein, niacin-deficient diet developed a normocytic anemia which responded to either protein or

niacin therapy. The anemia did not occur when the low-protein diet was supplemented with niacin throughout the experimental period.

Since synthesis of niacin from tryptophan has been demonstarted in intact animals (Singal et al., '46; Rosen et al., '46) and in tissue slices (Hurt et al., '49), it may be assumed that this conversion occurs in the monkey, although at the present time only indirect evidence is available to support the hypothesis. The wide occurrence in animals (Snell and Wright, '50) of such a synthetic mechanism lends further support to the conjecture that this also occurs in monkeys, although a comparison of the conversion ratios indicates wide species differences in the reaction mechanism or in other factors influencing this transformation.

SUMMARY

Rhesus monkeys receiving a niacin-deficient, purified diet containing 9% casein lose weight and develop lowered hemoglobin values after periods of several months.

Tryptophan has been shown capable of replacing the dietary niacin required by monkeys. A very inefficient conversion of tryptophan to niacin, however, occurs in this species.

The niacin requirements of monkeys receiving various diets are discussed.

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DIETARY FACTORS AFFECTING GASTRIC SECRETION IN THE RAT

EDGAR A. HAWK AND JAMES M. HUNDLEY

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Federal Security Agency, Bethesda, Maryland

TWO FIGURES

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INTRODUCTION

Komarov and co-workers ('44), using their pylorus-ligated fistula preparation, found no significant difference in gastric secretion between rats subsisting on a stock ration[1] and those on adequate purified diets. Madden et al. ('51), employing only pylorus ligation, reported equal volumes of secretion in rats on either type of diet when using an 18-hour secretory period, but noted smaller volumes in animals fed purified diets when a 6-hour period was employed.

We have observed consistent and marked differences in secretion between animals on the stock ration mentioned above and on purified diets, the difference being greatest in animals maintained for relatively long periods on these diets. The following experiments were conducted to investigate some of the factors responsible for these observations.

METHODS

Male weanling rats of the Sprague-Dawley strain (weight, 40 to 55 gm; age, 21 to 27 days) were obtained from the Na-

[1] Purina dog chow.

tional Institutes of Health breeding colony. The animals were housed individually in wire mesh-bottom metal cages with free access to food and water.

The accumulated gastric juice following pylorus ligation was used to measure gastric secretory activity. Our previously described procedure (Hawk and Hundley, '51) has been slightly modified from the method of Shay et al. ('45). Food was removed from the cage at about 5 P.M. so that the stomach would be empty on the following morning at about 9 A.M. when the pylorus was ligated under intraperitoneal sodium pentobarbital anesthesia (30 mg/kg), supplemented when necessary with ether. This overnight period without food was sufficient to empty the stomach in almost all cases.

At the end of the 6-hour secretory period (timed from operation) each animal was sacrificed, the stomach removed, and its contents expressed into a graduated centrifuge tube. The stomach was then opened and inspected. After the volume had been measured, the juice was centrifuged. Portions of the clear supernatant were used for titration of free and total acid concentration (dimethylaminoazobenzene and phenolphthalein, respectively) and determination of pepsin concentration by the milk clotting method of Saemundsson ('48). Crystalline pepsin [2] was used as a reference standard. Animals were discarded for any one of the following reasons: hemorrhage, perforation of an ulcer either into the peritoneal or pleural cavity, excessive food residue [3] in the juice, or grossly bloody juice.

The diets used were the stock ration [4] and a "complete" purified diet containing "vitamin-free" casein 20%, sucrose 72%, cottonseed oil [5] 3%, Osborne and Mendel ('17) salts 4%,

[2] Amour and Co.

[3] Animals were rejected if food residue exceeded 0.5 ml, except in the case of animals fed the diets containing wheat bran, where those with up to 0.6 ml of food residue were included. In almost all cases, food residues ranged from 0.1 to 0.3 ml.

[4] See footnote 1, page 87.

[5] Wesson.

and a vitamin mixture 1%.[6] In addition, the purified diet was modified by the inclusion of 5% whole dried liver powder [7] at the expense of sucrose to cover any unrecognized vitamin deficiency, and 9% non-nutritive fiber [8] at the expense of the whole diet, to simulate the fiber content of the stock diet.

In the first series of experiments, groups of animals were tested after they had been on these diets for periods ranging from two to 14 weeks, to determine the effect of diet on gastric secretion in relation to age and growth. Since the weight range of the animals used was very wide, correlation of secretory activity with body size was highly desirable. Thus, volume of secretion was expressed as milliliters per 100 gm body weight (per 6 hours), which is essentially the same as the previously used milliliters/100 gm/hour (Shay et al., '45). In addition, volume of secretion was related to body surface area as milliliters/100 cm^2 body surface [9] (per 6 hours).

Because of the large number of animals involved, it was not feasible to use the litter-mate control technique in these experiments. However, other groups of animals on stock and purified diets for three and 8 weeks were compared, using this technique, to determine as quantitatively as possible the relative secretory capacities of such animals.

In the second series of experiments a standard three-week feeding period was adopted to minimize the effects of age and body size, and strict litter-mate controls were used throughout. The "complete" purified diet was modified in various ways in an effort to increase the secretory performance of animals on this type of diet to a level comparable to

[6] Containing, in milligrams per gram, thiamine 0.4, riboflavin 0.6, pyridoxine 0.5, Ca pantothenate 4.0, choline chloride 200, inositol 20.0, nicotinic acid 4.0, 2-methyl naphthoquinone 0.2, biotin 0.02, and folic acid 0.2. Each animal received as supplements vitamin A 2,000 units and vitamin D 400 units twice weekly, and tocopherol 3 mg once weekly.

[7] Wilson and Co.

[8] General Biochemicals, Inc.

[9] Calculated according to the formula of Diack ('30) as surface area = $kW^{2/3}$, using k = 7.5.

that reached by stock diet animals. Protein was altered by substitution of blood fibrin at the same level or by changing the level of casein to 10% or 40% at the expense of sucrose. Carbohydrate was modified by substitution of either cornstarch or enriched white wheat flour for the entire sucrose content of the diet. Fat was changed by increasing the level of cottonseed oil from 3 to 10% at the expense of sucrose and by substituting butterfat at each of the two levels. The following substances were added individually at the specified percentages to the "complete" purified diet: dried brewers' yeast 5, corn bran 10, wheat bran 5, 10 and 20, dried defatted wheat germ 3, alpha-tocopherol acetate 20 mg %, defatted soy bean meal 10, and skim milk powder 10. In addition, the following [10] were added to the purified diet in the same manner: fish solubles 3, meat meal 10, dried beet pulp 10, dried figs 3, wheat cereal 10, and corn grits 10.

RESULTS

Figures 1 and 2 summarize in part the results obtained from the first series of experiments using animals fed the stock ration and the "complete" purified diet. Growth and general appearance were very similar for animals fed either diet. Pepsin concentration was essentially constant over the entire age-weight range for animals fed both types of diets. Acid concentration was always relatively high in animals having large volumes of secretion (in relation to body weight) and lower in those having smaller volumes. The extreme range of the means for "free" acid concentration was 87 to 129 milliequivalents/1.

The total volumes secreted by the stock diet animals (fig. 1) increased rapidly with increasing age and weight until the animals were about 50 days past weaning, and thereafter increased but little more. The irregularity in the latter part of the curve was due to the occasional animal which showed marked failure of secretion. When volume of secretion for

[10] Kindly supplied by Dr. W. C. Sherman of the Ralston Purina Co.

stock diet animals was expressed as milliliters/100 gm body weight, the curve declined with increasing age and size. However, when volume of secretion was expressed as milliliters/ 100 cm² body surface, a much flatter curve was obtained, indicating a more constant relationship of gastric secretion to surface area than to body weight over the entire range.

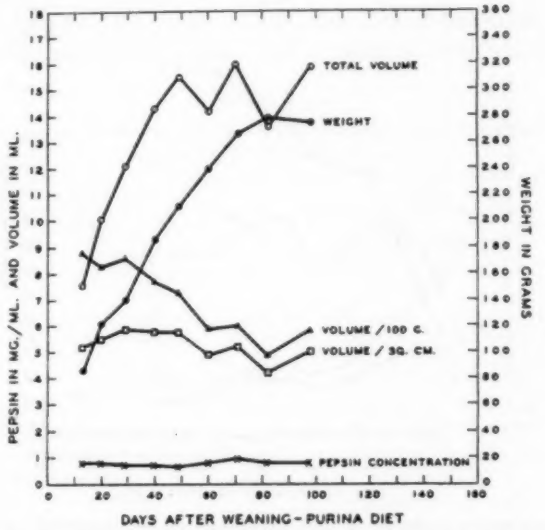

Fig. 1 Gastric secretion in rats subsisting on purina diet. Purina diet = stock diet. Each point on the curves represents the mean of 10 animals except in a few groups where operative and other casualties reduced the number to no fewer than 7. The probable error of the mean for both milliliters/100 gm and milliliters/100 cm² ranged from 0.1 to 0.3, except for the 80-day group, which had a probable error of 0.4.

In marked contrast, the volume of secretion for the purified diet animals was much lower over the entire age-weight range. The total volume actually increased but little with the marked increase in body size. The curve for milliliters/ 100 gm fell during the first 50 days past weaning and then tended to flatten out at a level considerably below that for comparable stock animals. As in the case of the stock diet

animals, the milliliters/100 cm^2 curve was much flatter than the milliliters/100 gm body weight curve, again indicating a more constant relationship of gastric secretion to body surface area than to body weight. In a similar series of experiments using animals fed the purified diet modified with liver

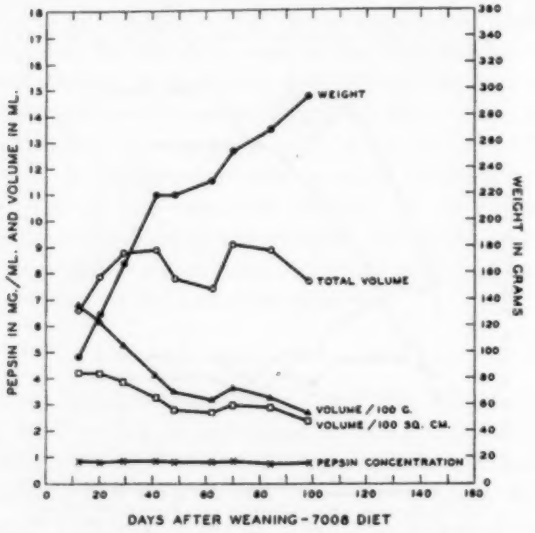

Fig. 2 Gastric secretion in rats subsisting on a "complete" purified diet. 7008 diet = "complete" purified diet. Each point on the curve represents the mean of 10 animals, except in a few groups where operative and other casualties reduced the number to no fewer than 7. The probable error of the mean for both milliliters/100 gm and milliliters/100 cm^2 ranged from 0.1 to 0.3, except for one group which had a probable error of 0.4.

powder or with non-nutritive fiber, the results essentially paralleled those for the purified diet animals.

These results made it obvious that animals fed a purified type of diet secreted considerably less over a wide age-weight range than did animals on a stock diet. This fact was further substantiated by the data given in table 1, which were obtained from strict litter-mate controlled experiments using animals which had been on the stock and purified diets for three and

for 8 weeks. The differences between volumes of secretion for stock and purified diet animals were statistically highly significant.[11] Additional experiments using rats fed a different type of stock diet [12] gave essentially the same results as those obtained with the stock diet mentioned above, at least up to about 6 weeks after weaning, the longest period during which these two diets were compared.

In the second series of experiments, modifications of the purified diet using whole wheat flour, wheat bran, wheat germ

TABLE 1

Volume and acid concentration of juice obtained from stock and "complete" purified diet animals

DIET	NO. OF ANIMALS	WEEKS PAST WEANING	WT.	JUICE			
				ml per 100 gm	ml per 100 cm²	Acid concentration	
						Free [1]	Total [1]
			gm				
Stock	18	3	103	8.6 ± 0.2 [2]	5.4 ± 0.1 [2]	116 ± 2 [2]	132 ± 2 [2]
Purified	18	3	111	6.1 ± 0.2	3.9 ± 0.1	102 ± 2	119 ± 2
Stock	7	8	238	6.3 ± 0.1	5.2 ± 0.1	115 ± 1	128 ± 1
Purified	9	8	224	3.6 ± 0.3	2.9 ± 0.2	94 ± 4	120 ± 3

[1] Milliequivalents/liter.

[2] Mean ± probable error.

or meat meal did produce increases in volume of secretion (table 2), although enriched white wheat flour did not. Although these increases were statistically significant, the observed volume of secretion did not quite equal that of comparable stock diet animals. The composition of the juice was essentially unchanged. Food residues in the juices from the wheat bran animals were relatively large.

Variations in the protein, carbohydrate, and fat of the purified diet had no significant influence on volume or composition

[11] Statistical significance of difference was determined according to the test procedure described in *Chemistry of Food and Nutrition*, Henry C. Sherman, The Macmillan Co., New York, N. Y., 1946, 7th ed.

[12] Hunt Club dog meal, manufactured by the Animal Foundation, Inc., Sherburne, N. Y.

of secretion, nor did additional tube-fed supplements of B vitamins [13] for two days prior to the operative procedure. An additional source of vitamins in the form of yeast, and corn bran as a source of roughage, likewise failed to affect secretion. No significant changes in secretory performance

TABLE 2

Volume of juice obtained from animals on various modifications of the "complete" purified diet

(All animals three weeks past weaning)

DIET	NO. OF ANIMALS	ML/100 CM²
"Complete" purified diet	48 [1]	3.7 ± 0.1 [2]
10% casein [3]	10	3.5 ± 0.1
40% casein [3]	10	3.6 ± 0.2
72% enriched white wheat flour [4]	9	3.9 ± 0.1
72% whole wheat flour [4]	9	4.4 ± 0.15
5% wheat bran [5]	7	3.9 ± 0.1
10% wheat bran [5]	8	4.7 ± 0.1
20% wheat bran [5]	8	4.4 ± 0.1
3% wheat germ [5]	7	4.3 ± 0.1
10% meat meal [5]	10	4.7 ± 0.15

[1] Including 5 individual groups, the means of which ranged from 3.6 to 3.8. The probable error of the mean for each individual group was 0.1 to 0.2.

[2] Mean ± probable error rounded to the nearest 0.05.

[3] Casein varied at expense of sucrose.

[4] Replacing sucrose.

[5] Added to the "complete" purified diet.

were observed in the animals fed any of the other modifications of the purified diet.

DISCUSSION

The reason for the increased volume in gastric secretion of rats on the stock diet as compared to that of rats on the purified diet cannot be fully explained at this time. However,

[13] Two individual supplements were given, each containing the following in milligrams, dissolved or suspended in water: thiamine 0.1, riboflavin 0.1, pyridoxine 0.1, nicotinamide 1.0, Ca pantothenate 1.0, folic acid 0.05, and vitamin B_{12} 5 μg. Controls were given a similar volume of water.

the effect of the addition of certain crude substances, including wheat products and meat meal, suggests that some factor affecting gastric secretion may have been deficient in the purified diet. It appears unlikely that the effect of these products was due to protein alone, since secretory performance was not altered in purified diet animals by variations in the amount of casein or by substitution of blood fibrin. The effect of the whole wheat flour was probably due to the contained bran or germ, since enriched white flour failed to increase the volume of secretion. In the case of wheat bran, roughage or bulk may be factors, even though we did not observe similar results in animals fed supplements of non-nutritive fiber or corn bran. The effect of the wheat products was apparently not due to vitamin E, since the addition of this vitamin to the purified diet did not change the volume of secretion. Preliminary experiments involving the feeding of either a water-alcohol extract or the residue from wheat bran have as yet been inconclusive. Carbohydrate, fat, and known vitamins do not appear to account for the difference between secretory performances by rats on the two diets.

In our smaller stock diet animals, the volumes of secretion were of the same general magnitude as those reported by Madden et al. ('51) using a much longer (18-hr.) secretory period. Pauls et al. ('47) have found that volume increases rapidly only up to about 6 hours after pylorus ligation. Cope et al. ('43) reported that water can be readily absorbed from the stomach. In view of these facts, it seems reasonable to assume that the volume of accumulated secretion found after a 6-hour period is more nearly a measure of secretory rate than is the volume of secretion after a longer period. A period shorter than 6 hours was not employed in order to minimize the effects of anesthesia and operative trauma.

Certain metabolic functions, such as oxygen consumption and food intake, can be correlated with body weight only

over a very limited range. Such metabolic functions can be correlated much more closely with body surface area. Since gastric secretion is a step in the process of preparing food for utilization by the animal, it is not surprising that this function can also be correlated more closely with body surface area than with body weight.

The antibiotic supplement currently included in commercial dog chow [14] may have some influence on gastric secretion. However, it does not explain the difference between the performances of stock and purified diet animals, since animals maintained on another type of stock diet [15] which did not contain an antibiotic secreted volumes approximating those of the commercial dog chow diet animals. In addition, aureomycin added to the purified diet with or without vitamin B_{12} did not alter secretory performance (Hawk and Hundley, '51). When aureomycin, 20 mg %, was added to the stock diet, the mean volume of secretion was increased but not quite to a statistically significant degree.

SUMMARY

Rats maintained on a stock diet secreted larger volumes of gastric juice in 6 hours following pylorus ligation than did those on a "complete" purified diet. The difference was relatively greater in animals maintained on the diets for longer periods of time. The inclusion of certain wheat products or meat meal in the purified diet increased the volume of secretion, although not up to the stock diet level. These findings suggest that the purified diet was deficient in some factor or factors affecting gastric secretion.

Volume of secretion per unit of body surface area is a more satisfactory expression than is volume per unit of body weight for the comparison of gastric secretory performance in pylorus-ligated rats over a wide age-size range.

[14] Purina.

[15] See footnote 12, page 93.

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EFFECT OF AUREOMYCIN AND PENICILLIN UPON THE VITAMIN REQUIREMENTS OF THE RAT [1]

H. E. SAUBERLICH

Department of Animal Husbandry and Nutrition, Agricultural Experiment Station of the Alabama Polytechnic Institute, Auburn

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Recent reports by Stokstad and Jukes ('50, '51) and by Oleson et al. ('50) indicate that aureomycin has a sparing effect upon the vitamin B_{12} requirement of the chick and turkey poult. This is in marked contrast to the results of earlier studies with other vitamins on the effect of the sulfonamides in the diet (Najjar and Barrett, '45; Schönheyder, '46; Elvehjem, '48). The present investigation was undertaken to determine whether or not similar antibiotics would have a sparing effect upon the vitamin requirements of the rat. During the progress of the present study, Linkswiler et al. ('51) reported on the effect of aureomycin on the response of rats to the various forms of vitamin B_6. Swick et al. ('51) also reported a similar study on the effects of antibiotics on the requirements for certain other vitamins by the rat.

EXPERIMENTAL

Weanling rats of either the Rockland strain (RS, male) or the Alabama Experiment Station strain (AES, mixed-sex) were placed individually in wire-bottomed cages. Food and

[1] Published with the approval of the Director of the Alabama Agricultural Experiment Station. The study was supported in part by a grant from the Society of the Sigma Xi. The antibiotics used in this study were generously furnished by the Lederle Laboratories Division, American Cyanamid Company (aureomycin), Merck and Company, Inc. (penicillin), and Sharp and Dohme, Inc. (sulfamerazine). The folacin was also donated by the Lederle Laboratories and all other vitamins were supplied by Merck and Company, Inc.

water were given ad libitum and the animals were weighed at weekly intervals. Four rats were used per group and nearly all experiments were repeated one or more times. The animals were fed the basal diet or a modification of it, as indicated, for a period of 4 weeks. The basal diet had the following percentage composition: methanol-extracted casein 20, corn oil 4, cod liver oil 1, carbohydrate 70.5, salts [2] 4, L-cystine 0.3, choline chloride 0.2. The following vitamins were added, in milligrams per kilogram of diet: α-tocopherol 25, α-tocopherol acetate 25, 2-methyl-1,4-naphthoquinone 2, inositol 1,000, niacin 20, calcium pantothenate 30, thiamine 6, riboflavin 6, pyridoxine 6, folacin 1, biotin 0.5, and B_{12} 0.03.

The modifications of the basal diet consisted of: (1) the omission of, or the controlling of the level in the diet of, the vitamin under study, which was pyridoxine, riboflavin, thiamine, or pantothenic acid; (2) using sucrose, dextrin, or starch as the carbohydrate in the diet; and (3) the addition of, or omission of, antibiotics to the modified diets. The antibiotics employed were crystalline aureomycin HCl, crystalline penicillin (G), or sulfamerazine. The aureomycin and penicillin were added to the diets at a level of 0.01%, and sulfamerazine was added at levels of 0.5 to 1.0%.

RESULTS

The presence of aureomycin or penicillin in the fully supplemented basal diet had no effect upon the growth of the rats (table 1). However, the addition of sulfamerazine, even at a level of 0.5% of the diet, had an inhibitory effect upon growth. The omission from the diet of thiamine, riboflavin, pyridoxine, or calcium pantothenate caused a marked reduction in the growth rate of the weanling rats during the 4-week period (tables 2 through 5). The effect of the omission of these vitamins was significantly altered by the presence of antibiotics in the diet. This effect appeared to be somewhat

[2] Salmon, W. D., J. Nutrition, *33:* 155, 1947.

dependent upon the vitamin and antibiotic under study, and was also influenced by the carbohydrate source in the diet.

Thiamine deficiency

When thiamine was omitted from the sucrose basal diet, a 50% mortality in the Rockland strain of rats was observed, with little or no increase in the weight of the survivors at the end of the 4-week period (table 2). Upon the addition to the diet of 0.01% of aureomycin, the mortality was eliminated and a small amount of growth was obtained in the rats. The addition of penicillin to the diet had an even more pronounced

TABLE 1

Effect of antibiotics upon the growth of rats

(AES strain of rats)

DIET	AVERAGE WEIGHT AT		
	0 wk.	2 wk.	4 wk.
	gm.	*gm.*	*gm.*
Sucrose basal	45	103	170
Sucrose basal + 0.01% aureomycin	45	105	168
Sucrose basal + 0.01% penicillin	46	111	172
Sucrose basal + 0.5% sulfamerazine	44	82	108
Sucrose basal + 1.0% sulfamerazine	46	65	77

effect; growth about 20% of normal at 4 weeks was actually obtained. The addition of sulfamerazine to the diet had no effect upon the thiamine deficiency.

When a suboptimum amount of thiamine was added to the diet (250 μg/kg), the influence of penicillin in the diet was even more marked. This level of thiamine permitted about 10% of normal growth at 4 weeks in the absence of penicillin and 47% in its presence (table 2). The effect of the antibiotics on thiamine deficiency was most striking when dextrin was substituted for sucrose in the diet. The dextrin-thiamine-free basal diet even permitted 9% of normal growth, but upon

the addition of aureomycin or penicillin, 48% and 68% of normal growth, respectively, was obtained (table 2).

When the AES strain of rats was used in place of the Rockland strain similar results were obtained (table 2), indicating

TABLE 2

Effect of antibiotics upon the thiamine requirement of the rat

DIET	AVERAGE WEIGHT AT			% OF NORMAL GAIN IN 4 WEEKS
	0 wk.	2 wk.	4 wk.	
	gm	*gm*	*gm*	
Rockland strain rats				
Sucrose basal [1]	47	108	177	100
Sucrose basal, thiamine omitted [2]	52	72	51	−1
Sucrose basal — B_1 + 0.01% aureomycin	53	66	59	5
Sucrose basal — B_1 + 0.01% penicillin	53	84	81	21
Sucrose basal — B_1 + 0.5% sulfamerazine [2]	52	68	50	−2
Sucrose basal 250 μg thiamine/kg diet	53	85	66	10
Sucrose basal 250 B_1 + 0.01% aureomycin	49	86	74	19
Sucrose basal 250 B_1 + 0.01% penicillin	55	102	117	47
Sucrose basal 250 B_1 + 0.5% sulfamerazine	47	77	65	14
Dextrin basal, thiamine omitted	52	79	64	9
Dextrin basal — B_1 + 0.01% aureomycin	53	95	115	48
Dextrin basal — B_1 + 0.01% penicillin	56	88	145	68
Dextrin basal — B_1 + 0.5% sulfamerazine	50	73	64	10
AES strain rats				
Sucrose basal [1]	47	114	195	100
Sucrose basal, thiamine omitted [2]	44	63	48	1
Sucrose basal — B_1 + 0.01% aureomycin	45	72	53	5
Sucrose basal — B_1 + 0.01% penicillin	50	89	80	20
Sucrose basal 250 μg thiamine/kg diet	45	69	45	0
Sucrose basal 250 B_1 + 0.01% penicillin	46	85	99	36

[1] Completely supplemented diet, containing 6 mg of thiamine per kilogram.

[2] A mortality of 50% obtained in 4 weeks.

that the effect of the antibiotics on the thiamine deficiency was not limited to a given strain of rats.

Riboflavin deficiency

The influence of aureomycin and penicillin upon the riboflavin deficiency was not as pronounced as it was in the case

of the thiamine deficiency (table 3). In fact, when the riboflavin-deficient sucrose basal diet was employed, no observable effect was noted when the antibiotics were added. However, when a suboptimum amount of riboflavin was present in this diet (250 μg/kg), the addition of penicillin had some influence. This level of riboflavin in the diet permitted about 30% of normal growth at 4 weeks in the absence of penicillin,

TABLE 3

Effect of antibiotics upon the riboflavin requirement of the rat

(Rockland strain rats)

DIET	AVERAGE WEIGHT AT			% OF NORMAL GAIN IN 4 WEEKS
	0 wk.	2 wk.	4 wk.	
	gm	*gm*	*gm*	
Sucrose basal [1]	47	108	177	100
Sucrose basal, riboflavin omitted	53	66	81	22
Sucrose basal — B_2 + 0.01% aureomycin	55	68	84	22
Sucrose basal — B_2 + 0.01% penicillin	51	68	76	19
Sucrose basal — B_2 + 0.5% sulfamerazine	54	65	62	6
Sucrose basal 250 μg riboflavin/kg diet	53	71	92	30
Sucrose diet 250 B_2 + 0.01% aureomycin	52	64	80	22
Sucrose basal 250 B_2 + 0.01% penicillin	50	76	104	42
Sucrose basal 250 B_2 + 0.5% sulfamerazine	47	57	57	8
Dextrin basal, riboflavin omitted	57	87	108	40
Dextrin basal — B_2 + 0.01% aureomycin	54	66	69	12
Dextrin basal — B_2 + 0.01% penicillin	51	77	104	41

[1] Completely supplemented diet, containing 6 mg of riboflavin per kilogram.

and 42% in its presence. Aureomycin appeared to be slightly inhibitory under these conditions. When dextrin was substituted for sucrose in the diet unsupplemented with riboflavin, the growth of the rats was nearly doubled (40% of normal on the dextrin diet at 4 weeks compared with 22% of normal on the sucrose diet; table 3). However, with the dextrin diet the addition of penicillin was without effect and aureomycin again appeared to be inhibitory. Similar results were obtained in experiments using the AES strain of rats.

Vitamin B_6 deficiency

Aureomycin was without marked influence upon the vitamin B_6 deficiency of the rat, whereas penicillin had a significant effect regardless of whether dextrin or sucrose was used as the carbohydrate source in the diet (table 4). Dextrin alone had a marked influence upon the vitamin B_6 requirement of the rat. Growth 65% of normal at 4 weeks was obtained when dextrin was present in the vitamin B_6-deficient diet, compared to growth 32% of normal when sucrose was employed. Moreover, when penicillin was added to the dextrin-vitamin B_6-free diet, growth of 85% of normal was obtained. Aureomycin appeared to be without influence in this diet and sulfamerazine was inhibitory. Experiments with the AES strain of rats revealed similar results (table 4).

Pantothenic acid deficiency

The presence of either aureomycin or penicillin in the pantothenic acid-deficient sucrose basal diet had a significant influence upon the growth of the rats (table 5). When calcium pantothenate was omitted from the basal diet, the rats gained in weight at a rate about 35% of normal at 4 weeks. Upon the addition of penicillin or of aureomycin to the diet, growths of 55% and 60% of normal, respectively, were obtained. Thus both aureomycin and penicillin appeared to have a marked influence upon the pantothenic acid requirement of the weanling rat.

DISCUSSION

On the basis of the present study, it is apparent that antibiotics may have a "moderating" or "sparing" effect upon the requirements of the weanling rat for certain vitamins. This effect appears to be somewhat dependent upon the vitamin under study. For instance, penicillin appeared to be more effective than aureomycin in the case of thiamine or vitamin B_6 deficiencies, whereas aureomycin and penicillin were equally effective in pantothenic acid deficiency. These results are in

general agreement with the findings reported by Linkswiler et al. ('51) and Swick et al. ('51). The present results with penicillin and aureomycin are in marked contrast to previous results with the sulfonamides, where requirements for certain vitamins were increased by their presence in the diet (Najjar and Barrett, '45; Schönheyder, '46; Elvehjem, '48).

TABLE 4

Effect of antibiotics upon the pyridoxine requirement of the rat

DIET	AVERAGE WEIGHT AT			% OF NORMAL GAIN IN 4 WEEKS
	0 wk.	2 wk.	4 wk.	
	gm	gm	gm	
Rockland strain rats				
Sucrose basal [1]	47	108	177	100
Sucrose basal, pyridoxine omitted	48	77	89	32
Sucrose basal — B_6 + 0.01% aureomycin	48	75	93	35
Sucrose basal — B_6 + 0.01% penicillin	48	79	101	41
Sucrose basal — B_6 + 0.5% sulfamerazine	52	69	70	14
Dextrin basal, pyridoxine omitted	53	105	138	65
Dextrin basal — B_6 + 0.01% aureomycin	54	92	129	58
Dextrin basal — B_6 + 0.01% penicillin	55	101	166	85
Dextrin basal — B_6 + 0.5% sulfamerazine	50	65	71	16
AES strain rats				
Sucrose basal [1]	45	103	170	100
Sucrose basal, pyridoxine omitted	37	57	65	22
Sucrose basal — B_6 + 0.01% aureomycin	41	64	75	27
Sucrose basal — B_6 + 0.01% penicillin	39	77	90	41
Starch basal — B_6	38	63	75	30
Starch basal — B_6 + 0.01% aureomycin	42	78	89	38

[1] Completely supplemented diet, containing 6 mg of pyridoxine per kilogram.

Several possible explanations of the mechanism by which antibiotics such as penicillin and aureomycin exert this action may be offered. Some of the more apparent of these are: (1) an inhibitory action of the aureomycin and penicillin upon microorganisms in the intestinal tract which normally utilize or absorb any traces of the vitamin in the diet, or that may have been produced by other organisms present which are

themselves unaffected by the added antibiotic; or (2) an altered intestinal flora due to the presence of the antibiotic which permits an increased amount of synthesis of the necessary vitamin; or (3) a combination of the effects of (1) and (2). In each instance, an increased amount of the essential nutrient would be made available to the host. Similar and additional explanations have been offered recently by other investigators (Stokstad and Jukes, '51; Waisman et al., '51; Linskwiler et al., '51). The effect obtained by substituting dextrin for sucrose in the diet can best be explained on the

TABLE 5

Effect of antibiotics upon the pantothenic acid requirement of the rat

(AES strain of rats)

DIET	AVERAGE WEIGHT AT			% OF NORMAL GAIN IN 4 WEEKS
	0 wk.	2 wk.	4 wk.	
	gm	*gm*	*gm*	
Sucrose basal [1]	45	108	162	100
Sucrose basal, calcium pantothenate omitted	45	73	86	35
Sucrose basal — P.A. + 0.01% aureomycin	45	89	115	60
Sucrose basal — P.A. + 0.01% pencillin	44	84	108	55

[1] Completely supplemented diet, containing 30 mg of calcium pantothenate per kilogram.

basis of the offering through this substitution of a more favorable medium for intestinal synthesis of the vitamins by the bacterial flora (Schweigert et al., '45; Teply et al., '46; Skeggs and Wright, '46; Couch et al., '48). The effect of the sulfonamides, on the other hand, is apparently brought about through the suppression of those microorganisms in the intestinal tract which are capable of synthesizing essential nutrients (Najjar and Barrett, '45; Schönheyder, '46; Elvehjem, '48).

Since the present study indicates that aureomycin and penicillin have a "sparing" effect upon the several vitamins studied, it is entirely possible that this effect may actually extend to other known and possibly undiscovered factors. The

increasing use of crude fermentation mashes and other similar materials containing antibiotics may require certain considerations to be taken into account in evaluating the use of bioassays for some of the vitamins. However, as Linkswiler et al. ('51) have pointed out in the case of vitamin B_6, the presence of an antibiotic in a substance being assayed for a given vitamin could "cause erroneous results as far as the amount present is concerned, but they would not necessarily be erroneous in terms of the physiological benefit to be expected."

SUMMARY

1. Studies were made on the effect of aureomycin and penicillin upon the growth of weanling rats fed diets deficient in certain B vitamins.

2. The addition of penicillin to the diet (0.01%) caused a marked stimulation in the growth of rats fed diets free of or low in thiamine, pyridoxine, pantothenic acid and, to some extent, riboflavin. Aureomycin (0.01%) had a similar effect in the case of pantothenic acid and thiamine deficiencies. The effect of the antibiotics on thiamine and pyridoxine deficiencies was evident whether sucrose or dextrin was used in the diet. The inclusion of penicillin or aureomycin in the completely vitamin-supplemented basal diet had no effect upon the growth of the animals.

3. Similar results were obtained whether the Alabama Experiment Station strain or the Rockland strain of rats was used.

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THE REPLACEMENT VALUE OF THE ALASKA FIELD PEA (PISUM SATIVUM) FOR HUMAN SUBJECTS [1]

NETTIE C. ESSELBAUGH, HAZEL C. MURRAY,[2] LAURINE W. HARDIE AND MARGARET M. HARD

Department of Home Economics, Washington Agricultural Experiment Stations, Institute of Agricultural Sciences, The State College of Washington, Pullman

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The work of various authors has shown that the protein efficiency of pea protein is relatively high for the rat but is greatly improved by methionine supplementation. Woods et al. ('43), Russell et al. ('46), Murray ('48), and Petersen et al. ('44) working with chickens, demonstrated that methionine was also a limiting factor when peas were used as the sole source of protein in growth experiments. Cox et al. ('47) found that the addition of methionine to a casein hydrolysate increased nitrogen retention for dogs and rats but not for human subjects. These workers interpreted their findings to indicate that the need for methionine is greater for fur-bearing animals than for humans.

Mitchell and Beadles ('50), using a nitrogen balance method, determined the digestibility and biological value of 6 protein sources (whole egg, egg albumin, beef, casein, wheat gluten, and peanut flour) on male adult and growing albino rats. They compared their results with those secured by Hawley and associates ('48), who had conducted a similar study using humans. The digestibility of the proteins was

[1] Scientific Paper *1054*, Washington Agricultural Experiment Stations, Institute of Agricultural Sciences, The State College of Washington, Pullman.

[2] Present address: Department of Home Economics, Agricultural Experiment Station, University of Hawaii, Honolulu.

comparable for all groups but the biological values were quite different for those proteins deficient in cystine-methionine (beef, casein, peanut flour) or in lysine (wheat gluten). The authors concluded that the cystine-methionine requirement of the adult rat is greater in relation to the requirements for other amino acids than for the growing rat or the adult human, while the lysine requirement is much less. The greater need for the sulphur-containing amino acids by the rat was attributed to the requirements for hair growth.

In the light of the above investigations, it seemed desirable to evaluate the replacement value and digestibility of pea protein for humans.

EXPERIMENTAL PROCEDURES

The egg replacement value of proteins (Murlin and Mattill, '38) was used to determine the relative value of the protein of the Alaska split pea with and without added DL-methionine. Methionine supplements were given in a water solution in equal amounts three times daily, totaling 1.5 gm per day during the supplemented pea regimen. Each subject served as her own control; the utilization of the pea protein was compared with the utilization of the egg for each subject.

Dietary regimen

The experimental diets were of three types, designated as (1) the egg diet, (2) the pea diet, and (3) the pea and methionine diet. There were two experimental periods for both the egg and pea diets, the egg preceding the pea in each case. Except for the first egg regimen, which was continued for 20 days, each of the dietary regimens was of 16 days' duration and was divided into 4 four-day metabolic periods. Following a vacation of 18 days, both the egg and pea regimens were repeated, making a total of 9 metabolic periods for the egg diet, 8 for the peas, and 4 for the pea plus methionine. Metabolic adjustment to each new dietary regimen for each subject was followed by determination of the 24-hour urinary nitrogen. A 4-day period was found adequate in every case.

The daily protein intake for the three regimens averaged 35.1 gm ($N \times 6.25$); egg diet 35.0, pea diet 34.2, pea and methionine 36.2 gm. The experimental food, egg or pea, supplied approximately 86% of the total protein. The protein level of 35 gm was selected as approximating the protein requirement for young adult women (Bricker, Mitchell and Kinsman, '45), resulting in a small negative balance so that maximum utilization of the available protein would be made. All three diets were approximately isocaloric by calculation; the necessary adjustment between the egg and pea diets was made by changing the amounts of carbohydrate and fat. Individual calorie needs were adjusted by the addition or subtraction of sucrose or fat so that subjects neither gained nor lost weight. An average ratio of carbohydrate to fat of 4.8:1 (range 4.7 to 5.0) was maintained throughout the entire experiment for all subjects. By calculation from food tables (Bradley, '42), protein contributed approximately 6%, fat 30%, and carbohydrate 64% of the total daily caloric intake, a distribution generally accepted as characteristic of the typical American diet. A 1:1 ratio for calcium and phosphorus was maintained on all diets by adjustment with calcium carbonate.

All foods for the entire experiment, except perishables such as lettuce, were purchased at the beginning of the study to insure, as far as possible, constant composition of the food. Fresh eggs were obtained at the College Poultry Department from a confined flock of hens fed the same ration throughout the study. Likewise a representative sample of peas was taken from a large storage bin.

The egg or pea were fed three times daily. Both foods were served in the cooking dish in which they were prepared. From the well-mixed total amount of whole egg needed for the day, the individual daily allowances were weighed. The eggs were scrambled with washed butter; the peas were simmered in distilled water until tender.

The experimental diet was divided into the basal and the experimental protein foods. The basal diet, composed of low

protein foods,[3] was provided by 4 different menus repeated every 4-day metabolic period. A cornstarch cookie [4] carried a large proportion of the calories and the necessary added minerals.[5] To insure an adequate and constant amount of vitamins in a semi-synthetic diet, two vitamin supplements, a vitamin B complex product [6] and a fish liver oil,[7] were provided. By calculation, the experimental diet was planned to provide all nutrients, except protein, in amounts commensurate with the 1948 recommendations of the National Research Council (table 1). Each subject standardized her own distilled water intake, maintaining a constant intake throughout the experiment.

Choice of subjects

Six healthy young adult women ranging in age from 20 to 24 years were selected as subjects. Prior to the final selection of each, a medical examination was made to ascertain her

[3] Fresh lettuce and celery; canned grapefruit, peaches, applesauce, prune juice, grape juice, pears, plums, pineapple; frozen squash and strawberries; cornstarch cookie; washed butterfat and sucrose.

[4] A cookie recipe (Bricker et al., '49) was altered as follows:

	EGG DIET	PEA DIET		EGG DIET	PEA DIET
	gm	*gm*		*gm*	*gm*
Cornstarch	400	400	Salt mixture	44	44
Crisco	120	160	Calcium carbonate	1.73	0.94
Sucrose	120	95	Agar	5	5

[5] Salt mixture (Bricker et al., '49)

	%		%
$CaCO_3$	32.14	$NaHCO_3$	27.0
$KHCO_3$	50.18	$Ca(H_2PO_4)_2H_2O$	37.6
$MgSO_4 : 7H_2O$	13.78	Cornstarch	35.4
$FeC_6H_5O_7 : 3H_2O$	3.72		
$MnSO_4 : 4H_2O$	0.0472		
$CuSO_4 : 5H_2O$	0.1670		
KI	0.0094		

Baking powder

The salt mixture, as used, contained: 3.76 gm baking powder; 2.52 gm mineral salts; and 4.76 gm NaCl.

[6] Supplied through the courtesy of Eli Lilly and Company, Indianapolis, Indiana. Each capsule contained: thiamine chloride 5 mg; riboflavin 5 mg; ascorbic acid 75 mg; pyridoxine hydrochloride 2.5 mg; pantothenic acid 12.5 mg; nicatinamide 0.25 mg; and desiccated fraction of liver — stomach concentrate, 0.455 gm.

[7] Supplied through the courtesy of Eli Lilly and Company, Indianapolis, Indiana. The daily dose contained 3,300 U.S.P. units vitamin A and 660 U.S.P. units vitamin D.

physical health. This examination included a basal metabolism test; urinalysis; red, white, and differential cell counts; and measurement of hematocrit, hemoglobin, and sedimentation rates.

Descriptive tests

At the beginning of each period on the egg diet and at the end of every dietary regimen, a basal metabolism test (indirect calorimetry) was taken and the above blood picture was determined on fasting blood. Oral temperatures were

TABLE 1

Calculated daily mineral and vitamin content of the diets

	CALCIUM	PHOSPHORUS	IRON	VITAMIN A	VITAMIN B_1	VITAMIN B_2	VITAMIN C	NIACIN	VITAMIN D
	mg	*mg*	*mg*	*I.U.*	*mg*	*mg*	*mg*	*mg*	*I.U.*
				Egg diet					
Average 4-day period	970	970	14	9,714	5.4	5.9	169	27.3	400
				Pea diet					
Average 4-day period	825	825	14	3,370	6.2	5.6	171	31.2	400

taken twice daily. Body weight was determined daily just before lunch, without shoes or outdoor clothing. These observations were used to detect either the unanticipated effect of the experimental diet on the subject or possible illness. At the end of the experiment a final medical examination of the subjects was made.

Preparations of samples

Twenty-four-hour specimens of urine were collected under toluene. Carmine was used to separate the fecal material into the 4-day metabolic periods. An amount of basal food equal to one-half of that served to each subject was weighed

at the time of preparation as representative samples and put into a common food pool. The food and feces were heated on a steam bath, homogenized in a Waring Blendor with 20% hydrochloric acid and brought to a convenient weight with this acid.

Chemical analysis

The urine and test foods were prepared for methionine assay in the following ways:

To 50 ml of urine, 2 ml of concentrated hydrochloric acid were added. Samples were autoclaved 4 hours at 15 pounds' pressure. All samples were then neutralized with sodium hydroxide to a pH of 6.8 and diluted to 60 ml with distilled water, with the exception of samples collected during the period when methionine was given as a supplement. These were diluted to a volume of 75 ml, due to the increased amount of methionine in the samples.

Enzymatic hydrolysis of the peas and eggs was carried out by the procedure of Evans ('46), using pepsin, trypsin, and erepsin.

These digests were assayed microbiologically, using the organism *Leuconostoc mesenteroides* P-60 and the medium of Steele, Sauberlich, Reynolds and Baumann ('49).

The nitrogen of the food and excreta was determined by the macro-Kjeldahl method with the modification by Lauro ('31).

RESULTS AND DISCUSSION

Nitrogen balances for each subject on the three dietary regimens are shown in table 2. Average figures are given for the two egg, the two pea, and the pea plus methionine regimens. In all subjects, except J.S., the egg protein was slightly superior to the protein of peas as judged by nitrogen balances, but the average difference, using the "t" score method of statistical analysis (Snedecor, '46), was not significant (table 3). During the periods on the pea diet 5 subjects showed small negative balances, as contrasted with three during the periods on eggs. The average daily balance

TABLE 2

Average daily nitrogen balances on egg, pea, and pea plus methionine regimens

SUBJECT	INTAKE	URINE	FECES	TOTAL OUTPUT	NITROGEN BALANCE	APPARENT DIGESTIBILITY [1]	REPLACEMENT VALUE
	gm	gm	gm	gm	gm	%	%
			Egg regimen — Periods 1 and 3 [2]				
MS	5.55	4.94	0.85	5.79	— 0.24	84.7	....
JS	5.55	5.39	0.72	6.10	— 0.56	87.0	....
BO	5.72	5.09	0.51	5.60	+ 0.12	91.1	....
MP	5.61	4.40	1.03	5.43	+ 0.18	81.6	....
SG	5.53	4.30	1.01	5.31	+ 0.22	81.7	....
JL	5.66	5.26	0.73	5.99	— 0.33	87.1	....
Average	5.60	4.90	0.81	5.70	— 0.10	85.6	....
			Pea regimen — Periods 2 and 4 [3]				
MS	5.43	4.64	1.11	5.75	— 0.32	79.6	98.4 [4]
JS	5.43	4.76	1.07	5.83	— 0.40	80.2	100.0 [4]
BO	5.60	5.28	0.99	6.27	— 0.67	82.3	83.6 [4]
MP	5.46	4.38	1.06	5.44	+ 0.02	80.6	96.7 [4]
SG	5.43	4.16	1.36	5.52	— 0.09	75.0	93.6 [4]
JL	5.46	4.51	1.36	5.88	— 0.42	75.0	98.1 [4]
Average	5.47	4.62	1.16	5.78	— 0.31	78.8	95.1 [4]
			Pea + methionine regimen — Period 5 [5]				
MS	5.89	3.85	1.16	5.01	+ 0.88	80.3	100 [4] (118.8) [6]
JS	5.89	4.27	1.09	5.36	+ 0.54	81.5	100 [4] (118.5) [6]
BO	6.07	4.52	0.81	5.33	+ 0.74	86.6	100 [4] (110.4) [6]
MP	5.96	4.14	1.42	5.56	+ 0.39	76.2	100 [4] (103.5) [6]
SG	5.90	4.23	1.36	5.59	+ 0.31	76.9	100 [4] (101.5) [6]
JL	6.01	4.27	1.31	5.58	+ 0.42	78.2	100 [4] (112.6) [6]
Average	5.95	4.21	1.19	5.41	+ 0.55	80.0	100 [4] (110.9) [6]

[1] Uncorrected for metabolic fecal nitrogen.

[2] A total of 7 four-day metabolic periods.

[3] A total of 6 four-day metabolic periods.

[4] Calculated according to the formula of Murlin and Mattill ('38).

[5] A total of 3 four-day metabolic periods.

[6] Replacement value $= 100 + \left[\frac{(\text{Pea + methionine } N_2 \text{ balance}) - (\text{egg } N_2 \text{ balance})}{\text{Dietary pea + methionine } N_2} \times 100\right]$.

for all subjects during egg regimens was — 0.10 gm of nitrogen, in contrast to — 0.31 gm during the periods when peas were consumed.

The addition to the pea diet of 1.5 gm of DL-methionine daily was selected in view of recent findings (Rose, '49) with respect to the methionine requirements of man. Rose tentatively places the minimum daily requirement of L-methionine as being 1.1 gm, with a recommended daily intake of 2.2 gm, stating that D- and L-methionine are equally effective in the maintenance of nitrogen equilibrium in man. In the present study the peas contributed 0.56 gm of methionine per day, the peas and methionine, 2.06 gm, and the eggs, 1.03 gm per

TABLE 3

Statistical analysis of differences between nitrogen balances

NITROGEN BALANCES COMPARED	MEAN DIFFERENCE	"t" [1]
Pea vs. egg	0.21	1.75
Pea + methionine vs. egg	0.65	4.33 [2]
Pea + methionine vs. pea	0.86	7.17 [2]

[1] Calculated from Fischer's formula (Snedecor, '46).

[2] Significant at the 1% level.

day. These amounts do not include the negligible amount of methionine supplied by the basal diet. Methionine added to the peas markedly affected the nitrogen retention. On the pea plus methionine diet, all subjects maintained a positive balance, averaging + 0.55 gm of nitrogen daily. When this value was compared with those obtained by feeding either egg or pea, the differences between nitrogen balances were found to be significant at the 1% level.

Pea protein was found to be 95.1% (range, 83.6 to 100) as efficient as egg protein. This value is higher than the egg replacement values reported for other plant proteins. Murlin, Nasset and Marsh ('38) reported a range from 57 to 87% for the replacement value of the protein of breakfast cereals (wheat and oats). Similarly, Sumner, Pierce and Murlin

('38) reported an average value of 85% for wheat endosperm. These authors secured a figure of 82% for fresh milk and 85% for dry milk. Our egg replacement value of 95% indicates that split peas are a protein food of high biological value for human consumption.

With the positive nitrogen retention of all subjects upon the addition of methionine to the peas, the egg replacement value was increased to 100% as defined by Murlin and Mattill ('38). Recognizing the higher nitrogen retention on the pea plus methionine diet, the replacement value could be expressed as over 100%, since the utilization is greater than that of egg protein. The average replacement value of 110.9% (shown in parentheses in table 2) emphasizes the superiority of the pea and added methionine when the utilization of this combination is compared with the utilization of the protein of egg.

Sumner, Pierce and Murlin ('38) pointed out that replacement values can be varied, depending on the amount of dietary protein. They showed that a higher intake of protein may conceal the fact that the experimental protein is low in some essential amino acid. Using young men as subjects, at the two dietary levels of 3.5 and 6.5 gm of nitrogen daily, they obtained replacement values of 82 and 100%, respectively, for milk. The increase in replacement value from 95 to 100% or higher resulting in the present study from the supplementation of peas with methionine indicates that the relatively low amount of this amino acid in peas was not masked by the level of dietary protein fed. Murlin, Nasset and Marsh ('38), in determining the egg replacement value of cereals, provided a daily nitrogen intake of 5.9 gm, which is comparable to the daily nitrogen intake of 5.6 gm in this study. This represents 5% and 6% of the total calories in the respective studies. The replacement values in these two experiments should be comparable and further support the finding as to the high replacement value of the split pea (95%) as compared with that of cereals (57 to 87%).

Egg protein was found to be slightly more digestible than pea; the average apparent coefficient of digestibility for egg was 85.6 and for pea 78.8% (table 2). Armbruster and Murray ('51), using rats, obtained a figure of 70.7% for canned peas and 76.0% for raw peas. For humans, Murlin Nasset and Marsh ('38) reported an apparent digestibility of 66% to 85% for the proteins of breakfast cereals. Hence, the digestibility of the protein of the split pea appears to be comparable to that of cereals. In this study, the coefficient of digestibility remained relatively constant with the addition of methionine, being 78.8% on the pea regimen and 80.0% on the pea plus methionine.

Cox et al. ('47) found that the addition of methionine to a casein hydrolysate facilitated nitrogen retention in dogs and rats but not in humans. They attributed this to a species difference in the requirement for the sulphur-containing amino acids. Likewise, Johnson and co-workers ('47), working with male adult human subjects, did not observe a decreased urinary nitrogen excretion when 3 gm of methionine were added either to low protein diets supplying 2 gm of nitrogen or to diets providing 12 gm of nitrogen daily. In the present study with young adult women there was an increased nitrogen retention following the addition of 1.5 gm of DL-methionine to the pea diet containing 5.5 gm of nitrogen daily.

The results of the methionine assays are shown in table 4. The addition to the peas of 1.5 gm of DL-methionine doubled the total ingested methionine when compared to the egg regime (1.03 mg to 2.06 mg) and provided approximately 4 times the amount ingested on the pea regime (0.56 gm to 2.06 mg, table 4). Methionine excretion was somewhat variable for all subjects; the average excretion for each person for both the egg and pea diet was about the same, although the eggs supplied about twice as much methionine as the peas. Excretion amounted to about 1.0% of the intake on the egg diet and 1.9% on the pea diet. Supplementation of the peas with the DL-methionine more than doubled the excretion, the

average value being 27.9 mg compared to 10.6 mg methionine per day. However, the per cent of ingested methionine excreted in the urine remained approximately the same as for the egg diet (1.4 to 1.0%). Steele, Reynolds and Baumann

TABLE 4

The dietary intake and urinary excretion of methionine

SUBJECT	INGESTED [1]	URINARY EXCRETION		EXCRETED
		Average	Range	
	gm/24 hrs.	*mg/24 hrs.*	*mg/24 hrs.*	%
		Egg diet		
MS	1.03	15.2	11.1–20.8	1.5
JS	1.03	9.7	7.9–12.5	0.9
BO	1.03	13.4	11.6–14.5	1.3
MP	1.03	10.8	10.1–11.1	1.0
SG	1.03	7.6	6.5–10.5	0.7
JL	1.03	7.5	6.7–10.6	0.7
Average	1.03	10.7		1.0
		Pea diet		
MS	0.56	12.2	10.6–13.9	2.2
JS	0.56	9.6	6.3–13.5	1.7
BO	0.56	14.1	10.3–14.9	2.5
MP	0.56	10.0	8.7–11.8	1.8
SG	0.56	8.0	7.5–13.0	1.4
JL	0.56	9.7	7.8–12.3	1.7
Average	0.56	10.6		1.9
		Pea + methionine diet		
MS	2.06	33.6	30.0–36.0	1.6
JS	2.06	27.5	23.2–30.5	1.3
BO	2.06	29.7	25.5–33.2	1.4
MP	2.06	24.0	20.7–27.1	1.2
SG	2.06	22.7	20.9–26.3	1.1
JL	2.06	29.9	23.0–33.8	1.5
Average	2.06	27.9		1.4

[1] Methionine in test food only.

('50), working with human subjects, found that urinary methionine was relatively constant when the same proteins (beef, milk, and bread) were fed at three different levels. The increase observed in the present experiment may be due to the fact that methionine was fed as the free amino acid.

The values obtained from blood analyses and basal metabolism tests were within the normal range throughout the study.

Within the range of error due to the small variation in weight of clothes, all subjects maintained their weight throughout the study. Maximum change between the initial and final weights of any one subject was 1.4 kg. It was necessary to increase the daily caloric intake from the initial 2,200 to 2,300 to maintain body weight for subjects M.S. and J.S. For subject S.G., the daily caloric intake was reduced from 2,200 to 2,050. The other three subjects were able to maintain a constant weight on 2,200 cal. daily

SUMMARY

1. The replacement value of the protein of the Alaska split pea *(Pisum sativum)* has been compared with that of egg protein. Six young adult women served as subjects.

2. Pea protein was slightly less efficient than egg protein for the human adult subject. The average daily nitrogen balance on the egg diet was — 0.10 gm, while that on the pea diet was — 0.31 gm. This difference was not statistically significant.

3. The egg replacement value of the pea protein was found to be 95.1%. This indicates that the split pea is a protein food of high biological value for human consumption.

4. When the daily methionine intake was increased to 2.06 gm by supplementation of the pea diet with 1.5 gm of DL-methionine, the egg replacement value for the pea protein was 100% or better for all subjects. If this replacement value is expressed in terms of the positive nitrogen balance obtained in contrast to that on the egg regimen, the average value becomes 110.9%.

5. The average apparent digestibility of egg was 85.6%, of peas 78.8%, and of peas plus methionine 80.0%.

6. Methionine supplements added to the pea diet increased nitrogen retention. The daily nitrogen balances of all subjects were positive during this period, resulting in an average value of + 0.55 gm. This utilization was significantly

better at the 1% level when compared with that obtained on either the egg or the pea diet.

7. The average urinary excretion of methionine for each subject was about the same during the egg and pea periods. When the amount of methionine was increased 4 times as a supplement to peas, excretion of this amino acid was approximately doubled.

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FOLIC ACID DEFICIENCY IN THE LAMB

HAROLD H. DRAPER AND B. CONNOR JOHNSON
Division of Animal Nutrition, University of Illinois, Urbana

ONE FIGURE

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The extensive study of the chemistry and physiological role of pteroylglutamates carried on over the past several years has included characterization of the folic acid deficiency state in several species. The generalized symptoms of folic acid deficiency in animals are diarrhea, glossitis, lesions of the gastrointestinal tract and anemia. Hematological abnormalities amendable by folic acid compounds include macrocytic anemia in the human (Spies et al., '45) and chick (Hogan and Parrott, '40), and leukopenia or granulocytopenia in the monkey (Day et al., '45), rat (Spicer et al., '42) and mouse (Franklin et al., '48).

As a part of a series of investigations of the vitamin requirements of the immature ruminant, the effects of maintaining newborn lambs on a diet deficient in folic acid or containing a folic acid antagonist are reported in this paper.

EXPERIMENTAL

A series of 10 lambs, one to three days of age, were placed on a "synthetic milk" folic acid-deficient basal diet with and without the addition of folic acid (pteroylglutamic acid) or formyl folic acid or one of the antimetabolites "x-methyl" folic acid and aminopterin (4-aminofolic acid). The basal diet was similar to that used previously in this laboratory for raising newborn calves and lambs; its composition is

given in table 1. The casein, minerals and cerelose were taken into solution in hot water made slightly alkaline with sodium bicarbonate. The lard and fat-soluble vitamins were homogenized into the solution, which was subsequently diluted to contain 19.5% total solids and after pasteurization was stored under refrigeration until used. The water-soluble vitamins were added in a 30% ethanol solution at the time of feeding,

TABLE 1

Composition of "synthetic milk" basal diet

CONSTITUENTS	AMOUNT
Casein ("vitamin-free"), %, dry basis	30
Lard, %, dry basis	30
Mineral mixture,[1] %, dry basis	6
Cerelose, %, dry basis	34
Water-soluble vitamins	
Thiamine hydrochloride	0.97 mg per liter
Riboflavin	1.95 mg per liter
Calcium pantothenate	2.92 mg per liter
Nicotinic acid	3.90 mg per liter
Pyridoxine	0.97 mg per liter
Choline	390.0 mg per liter
Biotin	0.02 mg per liter
Fat-soluble vitamins	
Vitamin A	3000 I.U. per liter
Vitamin D	300 I.U. per liter
Alpha-tocopherol	1.5 mg per liter
2-methyl-1,4-naphthoquinone	0.39 mg per liter

[1] For composition of mineral mixture see Johnson, James and Krider ('48).

and sulfathalidine was included at the rate of 3 gm per liter of diet. Chloromycetin was also administered to deficient animals in an attempt to reduce the incidence of pneumonia. When used, folic acid was provided at a level of 0.39 mg per liter (2 μg per gram dry matter). In addition to the vitamin supplement added to the diet, each animal was given 25,000 I.U. of vitamin A and 2,500 I.U. of vitamin D at the start of the experiment and at monthly intervals thereafter.

RESULTS

The characteristic feature of folic acid deficiency observed in this experiment was undifferentiated leukopenia. Those lambs receiving no folic acid in the diet, or receiving the basal diet plus an antimetabolite, underwent a depression of the total white cell count as compared to initial values or to those of positive controls, with no concomitant disproportion in the differential count. Animals having low leukocyte values appeared to be predisposed to pneumonia, which in some

TABLE 2

Growth and food consumption data for folic acid-deficient and control lambs

LAMB NO.	DIETARY TREATMENT	WEEKS ON DIET	INITIAL WEIGHT	AVERAGE DAILY GAIN	DRY MATTER CONSUMED
			lb.	*lb.*	*kg*
1	Basal + folic	8	6	0.27	10.0
2	Basal + folic	11	6.5	0.34	21.7
3	Basal	8	10	0.44	21.2
4	Basal	6	5.5	0.15	5.4 [1]
5	Basal + formyl folic	7.5	8	0.47	12.6
6	Basal	7	9.5	0.40	13.4
7	Basal + aminopterin	10.5	7	0.45	23.5
8	Basal + aminopterin	10.5	9	0.40	20.5
9	Basal + "x-methyl" folic	4.5	6.5	0.25	7.2
	Basal + "x-methyl" folic	7	6	0.12	7.0 [1]
10	Basal + aminopterin	5	19	0.23	10.7

[1] These animals contracted bronchopneumonia.

cases culminated in death. Diarrhea was also prevalent in the deficient animals. No discernible effect of the deficiency on the growth rate was observed, a result which is in agreement with that of Copping and Pond for the rat ('50), and a low white cell level in the blood was not associated with a depression of appetite (see table 2).

A graphic representation of the influence of several pteroylglutamates, or of their absence, upon leukocyte levels is shown in figure 1. Two lambs (1 and 2) receiving folic acid in the diet exhibited white cell counts of 7.2 and 5.5 thousands

per cubic millimeter of blood after 8 and 11 weeks, respectively, whereas in two lambs (3 and 4) receiving the basal diet alone, levels of 3.3 and 2.7 thousands were observed after 8 and 6 weeks, respectively. Both deficient animals

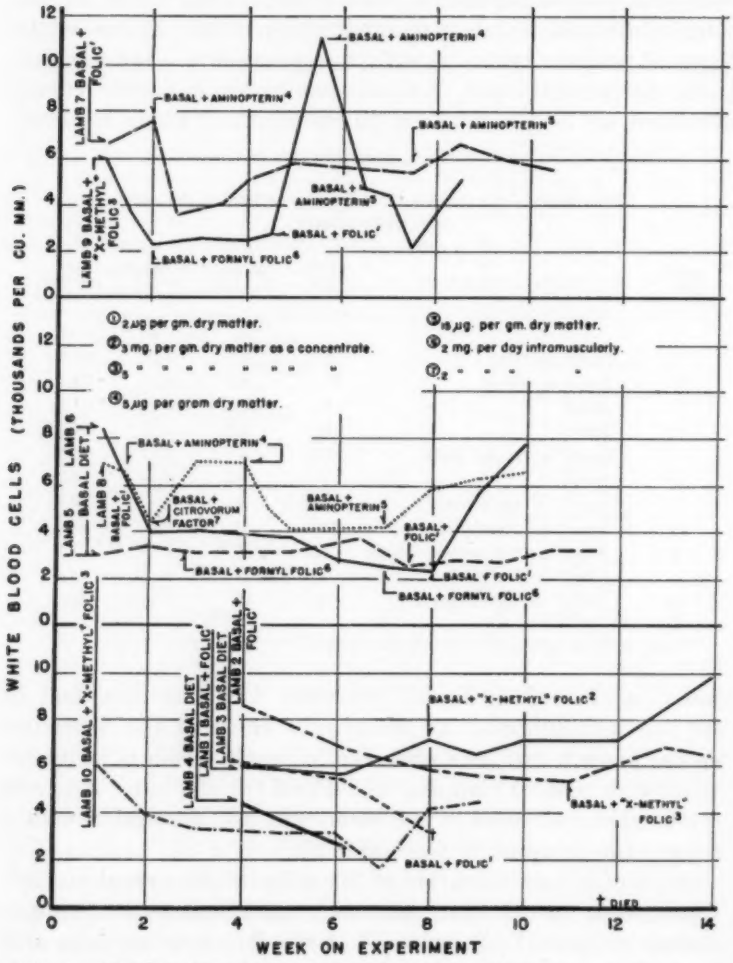
WHITE BLOOD CELLS (THOUSANDS PER CU. MM.)
WEEK ON EXPERIMENT
LAMB 7 BASAL + FOLIC¹
LAMB 9 BASAL + "X-METHYL" FOLIC³
BASAL + AMINOPTERIN⁴
BASAL + AMINOPTERIN⁵
BASAL + FOLIC¹
BASAL + FORMYL FOLIC⁶
① 2 µg per gm. dry matter.
② 3 mg. per gm. dry matter as a concentrate.
③ 5 " " " " " "
④ 5 µg per gram dry matter.
⑤ 15 µg. per gm. dry matter.
⑥ 2 mg. per day intramuscularly.
⑦ 2 " " "
LAMB 5
LAMB 6 BASAL DIET
LAMB 8 BASAL + FOLIC¹
BASAL + CITROVORUM FACTOR⁷
LAMB 10 BASAL + "X-METHYL" FOLIC³
LAMB 4 BASAL DIET
LAMB 1 BASAL + FOLIC¹
LAMB 3 BASAL DIET
LAMB 2 BASAL + FOLIC¹
BASAL + "X-METHYL" FOLIC²
† DIED

Fig. 1 Leukocyte levels in the blood of folic acid-deficient and control lambs.

died, and post mortem examination revealed pneumonia in one but no significant findings in the other.

After 7 weeks on the basal diet, the white cell count for lamb 6 was 2.6 thousands. Whereas supplementation with formyl folic acid at a level of 2 mg per day given intramuscularly failed to induce a leukocytosis, administration of folic acid was followed by an increase in leukocyte count to 5.8 thousands per cubic millimeter in one week and to 7.9 in two weeks. Thus, in this animal, as in lambs 5 and 9, formyl folic acid produced no significant hematological response, though in the case of lamb 5 no appreciable leukocytosis was induced by folic acid administration. The microbiological activity of the formyl folic acid was confirmed by assay with *Streptomyces faecalis R,* which indicated an activity for this organism approximately 80% of that of folic acid.

"X-methyl" folic acid was employed in the diet of 4 animals, in the basal diet of two from the beginning of the experiment (9 and 10) and in that of two others after 8 and 11 weeks, respectively, on the folic acid-supplemented basal (1 and 2). Leukopenia was observed only in the two animals receiving the antimetabolite from the start of the experiment. In one the white cell count was depressed to 1.7 thousands, the lowest level obtained under any treatment. Both animals responded satisfactorily to oral folic acid administration at a rate of 2 μg per gram dry matter in the diet. After 6 and three weeks of administration of the antivitamin, respectively, the leukocyte levels of lambs 1 and 2 were higher than at the start of treatment, indicating either a substantial storage of folic acid earlier in the experiment or a decreased requirement at the later age.

Aminopterin administration to three lambs (7, 8 and 9) at 5 μg per gram dry matter in the diet induced a similar result in each case. Following an initial sharp depression of the white cell count, which lasted for from two to 4 weeks, a gradual regeneration occurred, which persisted in spite of a threefold increase in the rate of aminopterin administration. The tolerance of lambs for these levels of this anti-

metabolite is in contrast to its lethal action in the case of the rat and mouse. The likelihood of a significant bacterial synthesis of folic acid in the intestine appears very remote in view of the acute thiamine deficiency obtainable in the lamb by employing a similar diet deficient in this vitamin (Draper and Johnson, '51). Moreover, the potency of folic acid in reversing aminopterin toxicity is known to be relatively slight. Microbiological assay of the feces of these animals was prevented, apparently by the presence of small amounts of chloromycetin. The adaptation of these lambs to substantial amounts of aminopterin may be comparable to the resistance displayed by leukemia patients exposed to prolonged therapy with this antimetabolite. In lamb 8 rapid leukocyte regeneration was obtained after aminopterin treatment by intramuscular administration of the "citrovorum factor" at a rate of 0.2 mg per day, indicating that this compound possesses biological activity for the lamb.

TABLE 3

Blood data for folic acid-deficient lambs and one positive control

LAMB NO.	2	3	10	11
DIETARY HISTORY	6 WKS. ON BASAL PLUS FOLIC	8 WKS. ON BASAL	2 WKS. ON BASAL PLUS AMINOPTERIN	7 WKS. ON BASAL PLUS "X-METHYL" FOLIC
Hemoglobin (gm/100 ml)	12.0	11.0	13.0	10.4
Erythrocytes (millions/mm^3)	6.7	7.2	7.6	6.4
Leukocytes (thousands/mm^3)	6.8	3.3	2.2	1.7
Neutrophils (%)	29	31	21	27
Lymphocytes (%)	53	64	76	72
Monocytes (%)			1	1
Eosinophils (%)	18	5	2	

Folic acid was determined in the urine of some animals by the *Streptococcus faecalis R* procedure of Rabinowitz and Snell ('47). Based on an average of three determinations per animal, the lambs receiving folic acid in the diet excreted 7 and 8 μg folic acid per day, respectively, over the first 8

weeks of the experiment. At the end of the experimental period the three lambs which had been receiving aminopterin in the diet for several weeks were excreting only 0.2, 0.3 and 0.5 μg of folic acid per day, respectively, even though a gradual leukocytosis was occurring concomitantly.

In table 3 are shown typical blood cell data for lambs deficient in folic acid as compared to a positive control. It is apparent that the only significant differences lie in the total white cell count.

Examination of the bone marrow of several deficient lambs indicated that abnormalities of the hyperplastic megaloblastic type found in human pernicious anemia were absent in these animals.

SUMMARY

A series of newborn lambs maintained on a "synthetic milk" diet deficient in folic acid developed a syndrome characterized by leukopenia and diarrhea, followed in most cases by pneumonia and death. White cell regeneration was induced by the administration of folic acid but no detectable response was obtained for formyl folic acid. "X-methyl" folic acid was effective as an antimetabolite when administered early in the experimental period and 4-amino folic acid produced a rapid depression in leukocyte levels, followed by a gradual regeneration which occurred in spite of increased doses of the antivitamin. The "citrovorum factor" was effective in reversing the depressive effect of aminopterin on white cell levels in the blood. Megaloblastic cells were absent from the bone marrow of folic acid-deficient animals. The results obtained demonstrate that folic acid is an essential nutrient for the lamb.

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FACTORS CONCERNED WITH THE DIFFERENT EROSIVE EFFECTS OF GRAPEFRUIT AND GRAPEFRUIT JUICE ON RATS' MOLAR TEETH

ROSS A. GORTNER, JR. AND REUBEN K. KENIGSBERG
Departments of Biology and Chemistry, Wesleyan University, Middletown, Connecticut

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INTRODUCTION

It has been known for many years that consumption of acid fruits and beverages may bring about appreciable damage to the teeth. McClure ('43) first used the rat for studying *in vivo* the enamel-erosive action of various soft drinks and fruit juices, and his observations were soon corroborated and extended by others (Restarski, Gortner and McCay, '45a; Gortner, Restarski, Bieri and McCay, '45; Bieri, McCay, Restarski and Gortner, '46; Wynn and Haldi, '48). Since these studies had all been conducted on beverages and juices, Miller ('50) decided to test the erosive effects of various acid fruits themselves, compared to those of the juices pressed therefrom. Her results were totally unexpected; namely, that the juices were three to 10 times more erosive than the equivalent amount of fresh fruit from which they were pressed. Although unable to explain these results, she pointed out that they were not due to differences in titratable acidity. Among the possible explanations which she listed were: (1) greater buffer action of the fruits themselves; (2) some protective factor in the fruit which is not contained in the juice; (3) some destructive constituent formed in the juice when it is removed from the fruit; and (4) a difference in physical contact with the teeth when the fruit and the juice are consumed.

In order to check Miller's results and investigate the explanation behind them, we have undertaken to study in detail the enamel-erosive properties of grapefruit and grapefruit juice, one of the combinations which Miller used.

EXPERIMENTAL PROCEDURES

For these studies Sherman-strain white rats bred in our own laboratory were used. They were placed on experiment at 8 to 10 weeks of age, at which time they weighed close to 200 gm. In each experimental group the animals were selected to be as similar as possible with respect to sex, age and weight. They were housed individually in raised-bottom wire cages.

All rats received a pelleted dog chow [1] in addition to the fresh grapefruit juice or fruit. The juice was presented in glass J-shaped drinking tubes, which minimized spillage or evaporation. The fruit sections (or the prepared artificial "fruit" described below) were fed in Syracuse dishes or Mason jar covers. By limiting, when necessary, the amount of dog chow or drinking water available to the animals, it was possible to get complete consumption of the fruit and juice.

Both the grapefruit sections and the juice were sweetened by the addition of 10% by weight of sucrose prior to feeding in order to make them more appetizing to the animals, since it has been shown (Muller and Gortner, '49) that inclusion of sugar does not influence the extent of acid erosion. The sweetened juice was consumed at a rate of 20 ml per day; fruit, on the other hand, was fed at a daily level of about 32 gm, corresponding in juice content to 20 ml. Two artificial "fruits" prepared from fresh sweetened grapefruit juice were also studied. In one instance the juice was heated to near boiling and 3% by weight of purified agar [2] was added. Upon cooling, a rather pasty solid with the consistency of very thick applesauce was obtained. In another instance

[1] GLF Big Red Dog Food, Canandaigua, N. Y.

[2] Bacto-agar, Difco Laboratories, Detroit, Mich.

small pieces of compressed, foamed gelatin [3] were added to the fresh juice; it was found that 1 gm of this gelatin soaked up 20 ml of the juice to form a soft, juice-filled sponge of about the same consistency as the natural grapefruit sections.

In order to assess the importance of salivation to the results obtained with grapefruit and grapefruit juice, 8 rats were anaesthetized by intraperitoneal injection of a 0.6% aqueous solution of nembutal (sodium pentobarbital). The dosage used was 4 mg nembutal per 100 gm body weight. Using sterile operational procedures described by Griffith and Farris ('42), the three pairs of salivary glands were carefully and completely excised and a small piece of thrombin-soaked gelfoam was inserted in the space formerly occupied by the glands to stop any bleeding prior to closing the incision. Recovery was rapid; the sutures were removed at the end of one week, and usually the animals were placed on experiment within two or three weeks.

After 7 days on the various supplemented diets, all animals were sacrificed and decapitated, the jaws were removed and cleaned, and the molars were scored as described previously (Restarski, Gortner and McCay, '45b). According to this procedure, the lingual aspects of the molars are examined under the low power of a dissecting binocular microscope and the extent of etching of each tooth is judged on a scale of zero (no effect) to 6 (extensive destruction). In all instances two observers independently recorded the extent of enamel erosion, one without knowledge of the supplement fed, with good agreement.

RESULTS AND DISCUSSION

The comparative erosive effects of fresh grapefruit sections and the juice pressed therefrom are shown in table 1. The data agree well with those reported by Miller ('50) and demonstrate clearly the greater destructive effect of the juice, as compared to the fruit, on the lower molars.

[3] Purified, de-ashed gelatin, Eastman Kodak Co., Rochester, N. Y.

In order to ascertain whether the different effects of fruit and juice might be due to some protective factor (buffer or otherwise) present in the fruit but absent in the juice, or some destructive agent formed in the juice on removal from the fruit, it seemed desirable to reconstitute some of the juice into a simulated fruit and compare the erosive effect of such a preparation with that of the natural fruit and juice. A 3% bacto-agar and grapefruit juice paste was first used in this connection. The agar was selected because it is an inert, non-digestible polysaccharide, as is the cellulosic supporting structure of fruit, it is essentially neutral in reaction and has little or no buffering power, and it dissolves fairly readily in hot juice. Although the rats readily consumed this

TABLE 1

Extent of enamel erosion of rats' molar teeth in 7 days by grapefruit and grapefruit juice

SUPPLEMENT	EQUIVALENT AMOUNTS FED DAILY	NO. OF RATS	AVERAGE TOOTH SCORES	
			Lower molars	Upper molars
Fruit sections	32 gm	25	1.5	1.0
Juice	20 ml	28	3.8	1.6

agar-juice preparation, it had certain disadvantages for comparison with fresh fruit. First, it necessitated heating the juice to a near-boiling temperature, which might conceivably destroy some heat-labile substance capable of influencing erosion; secondly, its pasty consistency was quite unlike that of the juicy fruit sections.

Accordingly a second artificial "fruit" was prepared from bits of pressed, foamed gelatin. This material readily soaked up the unheated juice to form a delicate juicy sponge of the desired consistency. Furthermore, its buffering effect was slight, as evidenced by the fact that the gelatin-juice and the juice alone had about the same pH.

Table 2 shows the results obtained with these simulated fruits. Both the agar and gelatin "fruits" behaved like the

fresh fruit sections in causing considerably less damage to the lower molar enamel than did the juice from which they were prepared. Thus the different extent of tooth damage wrought by the fruit and the juice cannot be explained on the basis of some protective substance present only in the fruit, or of some destructive agent arising during the process of pressing out the juice. It must be, therefore, that the differences are attributable to physical factors rather than to chemical differences.

It seemed a plausible hypothesis that the lessened damage caused by the "solid" fruits might result from increased salivary buffering. Mastication is known to stimulate mark-

TABLE 2

Erosive effects of grapefruit, grapefruit juice and two artificial "grapefruit" preparations when fed to rats for 7 days (All supplements were provided at a level equivalent to 20 ml of juice per day.)

SUPPLEMENT	AVERAGE pH	NO. OF RATS	AVERAGE TOOTH SCORES	
			Lower molars	Upper molars
Juice	3.5	28	3.8	1.6
Fresh fruit	3.5	25	1.5	1.0
Agar "fruit"	3.4	16	1.7	2.3
Gelatin "fruit"	3.6	13	1.0	0.8

edly the flow of saliva, so that in the process of chewing the fruit more of the acid might be buffered. To test this concept, 8 completely desalivated rats were pair-fed with litter mates of the same sex. Five of the pairs received fresh grapefruit sections, the other three received juice. Three of the pairs on the fruit supplement were continued on experiment for two weeks, since it was thought that differences between the operated animals and their controls would show up more clearly if a longer erosion period were permitted. The resulting data are presented in table 3.

No detectable difference in molar erosion was observed between the desalivated and control rats fed either the fruit or juice. Thus, in the rat at least, salivary buffering is appar-

ently unimportant in modifying the extent of tooth decalcification caused by citrus fruits or juices. This result is not altogether surprising in view of the considerable titratable acidity of grapefruit, equivalent to about 200 ml of 0.1 *N* sodium hydroxide per 100 ml of juice. Muller and Gortner

TABLE 3

Comparison of the enamel erosion caused in pair-fed desalivated and control rats by fresh grapefruit and grapefruit juice fed at a level of 20 ml of juice (or its equivalent) per day

SUPPLEMENT	ANIMAL	DAYS ON SUPPLEMENT	AVERAGE TOOTH SCORES	
			Lower molars	Upper molars
Juice	operated	7	3.7	3.5
	control		3.8	2.5
Juice	operated	7	4.4	1.2
	control		4.3	1.5
Juice	operated	7	3.8	1.9
	control		4.0	2.1
Fruit	operated	7	1.3	0.8
	control		1.0	0.7
Fruit	operated	7	1.8	1.8
	control		0.7	0.7
Fruit	operated	14	1.6	1.0
	control		2.0	1.2
Fruit	operated	14	2.0	1.1
	control		1.9	1.1
Fruit	operated	14	1.8	1.3
	control		1.7	1.2

('49) have previously reported that, whereas the rat can, during ingestion, effectively buffer a phosphoric acid solution of pH 3.3 and very low titratable acidity, it cannot appreciably buffer lactic acid solutions of even pH 3.5 to 4.0, because of their relatively high titratable acidity.

What explanation can be offered, then, for the different destructive actions of the fruit and juice? The answer must

lie in the different consistencies of the two supplements and the correspondingly different manners of ingestion. Probably the position and movement of the tongue during the drinking process expose the lingual aspects of the lower molars more completely to the acid juice than is the case during the eating of the fruit, when the base of the tongue would be retracted and more flattened against the molars.

That the consistency of the supplement may be a significant factor in enamel erosion is evident from the results obtained with the agar "fruit" (see table 2). With the juice, fresh fruit, and gelatin "fruit" the etching of the upper molars was, as anticipated (Gortner, Restarski, Bieri and McCay, '45), very mild as compared to the effect on the lower molars. The rats on the agar "fruit" diet, however, exhibited, almost without exception, greater damage to the upper molars than to the lower ones. It is believed that this result is attributable to the somewhat sticky nature of the agar mixture, causing it to adhere to the upper molars and thus permitting the acid to act on the enamel over a longer period of time. Under these circumstances the cleansing and shielding effects of the tongue could presumably protect the lower molars to the extent that they would be less severely attacked than the upper teeth.

SUMMARY

1. It has been confirmed that consumption of grapefruit juice causes considerably greater erosion of the lower molars of rats than does ingestion of grapefruit sections with similar juice content and pH.

2. When the pressed juice was reconstituted into a simulated "fruit" by the addition of 3% bacto-agar or 5% foamed, compressed gelatin, these preparations affected the teeth in about the same manner as the natural fruit and were much less destructive than the juice from which they were prepared. This implies that the differences are not due to some protective substance present only in the fresh unpressed fruit, or to some destructive agent arising in the juice during the squeezing process.

3. By pair-feeding 8 completely desalivated rats with litter-mate controls, it was shown that salivary buffering had no detectable influence on the extent of enamel erosion induced by either grapefruit or grapefruit juice, and could not explain the different destructive effects of the two supplements.

4. It is concluded that mechanical differences, such as the consistencies of the acid supplements and the different manners of ingestion, rather than chemical differences, are responsible for the effects noted.

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